

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau(43) International Publication Date  
25 July 2002 (25.07.2002)

PCT

(10) International Publication Number  
WO 02/057466 A2(51) International Patent Classification<sup>7</sup>: C12N 15/82,  
A01H 5/00(74) Agent: HARTZ, Nikolai, F.; Wächterhäuser & Hartz, Tal  
29, 80331 München (DE).

(21) International Application Number: PCT/EP02/00481

(22) International Filing Date: 18 January 2002 (18.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
101 02 389.8 19 January 2001 (19.01.2001) DE(71) Applicant (for all designated States except US): ICON  
GENETICS AG [DE/DE]; Maximilianstrasse 38/40,  
80539 München (DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): EIBL, Christian  
[DE/DE]; Sommerhaus, 85241 Herbertshausen (DE).  
HUANG, Fong-Chin [—/DE]; Frankfurter Ring 8, 80807  
München (DE). KLAUS, Sebastian [DE/DE]; Gräfstr. 34,  
81241 München (DE). MÜHLBAUER, Stefan [DE/DE];  
Ganzenmüllerstr. 8, 85354 Freising (DE). HERZ, Stefan  
[DE/DE]; Clemensstrasse 12, 80803 München (DE).  
KOOP, Hans-Ulrich [DE/DE]; Gerastrasse 11a, 80993  
München (DE).(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,  
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,  
VN, YU, ZA, ZM, ZW.(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,  
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent  
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,  
NE, SN, TD, TG).

## Published:

— without international search report and to be republished  
upon receipt of that reportFor two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

WO 02/057466 A2

(54) Title: PROCESSES AND VECTORS FOR PLASTID TRANSFORMATION OF HIGHER PLANTS

(57) Abstract: A process for producing multicellular plants, plant organs or plant tissues transformed on their plastome by the following steps is provided: (a) altering or disrupting the function of a gene in a plastid genome for producing a selectable or recognizable phenotype; (b) separating or selecting plants or cells having plastids expressing said phenotype; (c) transforming said plastid genome of said separated or selected plant, plant organ or plant tissue with at least one transformation vector having a restoring sequence capable of restoring said function; and (d) separating or selecting said transformed plant, plant organ or plant tissue having plastids expressing said restored function.

## Processes and Vectors for Plastid Transformation of Higher Plants

### FIELD OF THE INVENTION

The present invention generally pertains to plant molecular biology and more particularly pertains to novel methods for plastid transformation.

### BACKGROUND OF THE INVENTION

According to generally accepted knowledge, two classes of cell organelles, i.e. plastids and mitochondria, are derived from initially independent prokaryotes that were taken up into a predecessor of present day eukaryotic cells by separate endosymbiotic events (Gray, 1991). As a consequence, these organelles contain their own DNA, DNA transcripts in the form of messenger RNA, ribosomes, and at least some of the necessary tRNAs that are required for decoding of genetic information (Marechal-Drouard et al., 1991).

While, shortly after endosymbiotic uptake, these organelles were genetically autonomous, since they contained all the elements necessary to drive prokaryotic life, this autonomy was reduced during evolution by transfer of genetic information to the cell's nucleus. Nevertheless, their genetic information is of sufficient complexity to make recent cell organelles an attractive target for gene technology. This is particularly the case with plastids, because these organelles still encode about 50% of the proteins required for their main function inside the plant cell, photosynthesis. Plastids also encode their ribosomal RNAs, the majority of their tRNAs and ribosomal proteins. In total, the number of genes in the plastome is in the range of 120 (Palmer, 1991). The vast majority of proteins that are found in plastids are, however, imported from the nuclear/cytosolic genetic compartment.

#### *Plastids can be genetically transformed*

With the development of general molecular cloning technologies, it became soon possible to genetically modify higher plants by transformation. The main emphasis in plant transformation was and still is on nuclear transformation, since the majority of genes, ca. 26.000 in the case of *Arabidopsis thaliana*, the complete sequence of which was recently published (The Arabidopsis Genome Initiative, 2000), is found in the cell's nucleus. Nuclear transformation was easier to achieve, since biological vectors such as *Agrobacterium tumefaciens* were available, which could be modified to efficiently enable nuclear

transformation (Galvin, 1998). In addition, the nucleus is more directly accessible to foreign nucleic acids, while the organelles are surrounded by two envelope membranes that are, generally speaking, not permeable to macromolecules such as DNA.

A capability of transforming plastids is highly desirable since it could make use of the enormous gene dosage in these organelles – more than 10000 copies of the plastome may be present per cell – that bears the potential of extremely high expression levels of transgenes. In addition, plastid transformation is attractive because plastid-encoded traits are not pollen transmissible; hence, potential risks of inadvertent transgene escape to wild relatives of transgenic plants are largely reduced. Other potential advantages of plastid transformation include the feasibility of simultaneous expression of multiple genes as a polycistronic unit and the elimination of positional effects and gene silencing that may result following nuclear transformation.

Methods that allow stable transformation of plastids could indeed be developed for higher plants. To date, two different methods are available, i.e. particle bombardment of tissues, in particular leaf tissues (Svab et al., 1990), and treatment of protoplasts with polyethylene glycol (PEG) in the presence of suitable transformation vectors (Koop et al., 1996). Both methods mediate the transfer of plasmid DNA across the two envelope membranes into the organelle's stroma.

One significant disadvantage of all multicellular plant transformation procedures used today is the occurrence of marker genes in the transgenic plants. These marker genes that are needed for the selection of transgenic plant cells from a vast background of untransformed cells code for antibiotic or herbicide resistance genes. Examples for plastid resistance genes are *aadA* conferring resistance to spectinomycin and streptomycin (Svab & Maliga, 1993), or *nptII* conferring resistance to kanamycin (Carrer et al., 1993). As these marker genes are stably integrated into the genome together with the genes of interest (GOI), they will stay in the homoplastomic transgenic plants although they are not required for GOI function. These remaining marker genes are a main issue of criticism of plant biotechnology as they could theoretically increase antibiotic resistance of pathogens or herbicide resistance of weeds. Construction of a selection system which does not result in a resistance gene in the transgenic plant is, therefore, highly desirable (Iamtham and Day, 2000).

Another problem in plastid transformation is the shortage of selectable marker genes available. The *aadA* gene is the only selectable marker gene that is used routinely (Heifetz, 2000), and the *nptII* gene is the only alternative that has been shown to function in higher plant plastid transformation (Carrer et al., 1993). Since neither the *aadA* nor the *nptII* gene can be

used universally, the number of higher plant species that have been transformed in the plastome is still very low (Heifetz, 2000). Plastid transformation in higher plants cannot at present be exploited to its full potential.

#### SUMMARY OF THE INVENTION

It is an object of this invention to provide a simple, yet highly versatile process for producing genetically stable multicellular plants, plant organs or plant tissues transformed in their plastome, which are free of a foreign gene required for selection such as an antibiotic or herbicide resistance gene.

This object is achieved by a process for producing multicellular plants, plant organs or plant tissues transformed in their plastome by the following steps:

- (a) altering or disrupting the function of a gene in a plastid genome for producing a selectable or recognizable phenotype;
- (b) separating or selecting plants or cells having plastids expressing said phenotype;
- (c) transforming said plastid genome of said separated or selected plants, seeds, cells or plastids with at least one transformation vector having a restoring sequence capable of restoring said function; and
- (d) separating or selecting said transformed plants or cells having plastids expressing said restored function.

Preferred embodiments are defined in the subclaims.

It is surprising that this new method is readily applicable to multicellular plants, plant organs, or plant tissue since these contain a plurality of plastids in each cell, which means that segregation of genotypes is required on the level of plastomes, the level of plastids and the level of cells. It has been found that this new process is highly efficient for the tissue of higher plants since segregation occurs readily during growth. Separation is therefore simply possible by optical inspection and manual manipulation in appropriate embodiments. In cases of inhibitor-supported selection (step (b)), the selection process can be carried out rapidly since in the case of multicellular plants, plant organs or plant tissue the inhibitor does not need to be applied throughout the whole regeneration process, but may be applied only initially. (Of course, as explained above, it is possible to avoid inhibitors completely.) This shows a close combination effect between multicellularity and the method of transformation.

In the case of a transformation of a plant tissue by the process of the invention, the consequences of alteration or disruption of a gene, which may frequently be lethal in the case

of a single isolated cell if this gene is of central importance e.g. for a metabolic pathway, are mitigated by the fact that a single transformed cell does not stand in isolation. Rather, it is part of a population of cells among which metabolites may be exchanged.

There are many different plastid genes which can be altered or disrupted for the purposes of this invention. Such a gene should be important for plastid function in the sense of producing a selectable or recognisable phenotype upon alteration or disruption. Such a function may be any function which is plastid encoded. Preferably, this function is directly or indirectly involved in photosynthesis. Examples for functions indirectly necessary for photosynthesis are any functions needed for transcription and/or translation of plastid genes. Examples for functions directly involved in photosynthesis are any proteins which are essential, at least under selection conditions, for photosynthesis.

Preferably, said recognisable phenotype is easily discernable. Since said function above is preferably directly or indirectly associated with photosynthesis, an easily recognisable phenotype may be pigment deficiency, most preferably chlorophyll deficiency or altered fluorescence. The transformed plant may then be grown heterotrophically and transformed plants, plant organs or plant tissue may be separated or selected for. Separation may be effected manually by optical recognition of transformed tissue areas. Selection may be effected via inhibitor resistance based on a resistance gene introduced in step (a) of the process of this invention. Alternatively, inhibitor resistance may be a consequence of said altered or disrupted function itself.

After reaching the homoplastomic state by segregation during several rounds of regeneration, the transgenic plant, plant organ or plant tissue is transformed a second time (step (c)), whereby the altered or disrupted function is restored and the marker gene, if any, is removed. The transformed plants, plant organs or plant tissues having the restored phenotype, e.g. phototrophy, are subsequently separated or selected.

Additional sequences or genes of interest may be introduced in step (a) and/or step (c) e.g. for expressing a desired gene, for conferring a useful trait or for any other desired plastome modification.

Further, sequences introduced in step (a) and step (c) may together result in an additional function. Examples for this embodiment include the following: introducing sequences in step (a) and step (c) that code for different subunits of a multi-subunit protein; providing regulatory sequences in step (a) or step (c) that make a coding sequence introduced in step (c) or step (a), respectively, expressible; introducing sequences in steps (a) and (c) that code for proteins of a biochemical pathway; etc.

Specific examples of the function to be disrupted may be knock outs of *rpoA* or *rpoB*. These genes code for the  $\alpha$  and  $\beta$  subunit, respectively, of the plastid encoded plastid RNA polymerase. Plastids lacking these genes are not able to conduct photosynthesis, show an albino phenotype, and are not able to grow phototrophically. After restoration of *rpoA* or *rpoB* in the second round of transformation, the transgenic plants are able to grow phototrophically in light and show a green phenotype.

Another example of a target gene for step (a) may be a knock out of *ycf3*. This gene is not essential under normal light conditions (Ruf et al., 1997). Nevertheless, if a *ycf3* knock out mutant is placed under strong light, it develops an albino phenotype and growth is repressed under photosynthetic growth conditions. Plants with restored *ycf3* gene are able to grow phototrophically under strong light conditions. So in this case, the selection pressure for the second transformation can be adjusted by the light intensity. Therefore, in contrast to the example using *rpoA* or *rpoB*, the second transformation can be carried out with green, normally growing plant mutants when kept under low light conditions and selection pressure can be raised after a regeneration time simply by increasing the light intensity. As the condition of the plant material is critical for transformation, this method is superior to transformation of albino material.

Another example for the function to be altered or disrupted is a knock out of *petA* in the first round of transformation (step (a)). *petA* encodes a subunit of the cytochrome b/f complex. *petA* knock out mutants show a high chlorophyll fluorescence phenotype (hcf) and are not able to conduct photosynthesis. Therefore, they are not able to grow phototrophically. The phototrophic growth in light is restored, when *petA* is restored in the second round of transformation (step (c)).

## DEFINITIONS

The following definitions are given in order to clarify the meanings of certain terms used in the description of the present invention.

<b>3'-UTR</b>	transcribed but not translated region of a (-) <b>gene</b> , downstream of a (-) <b>coding region</b> ; in (-) <b>plastid (-) genes</b> , the <b>3'-UTR</b> a.o. serves to stabilise the mRNA against 3' to 5' exonucleolytic degradation
<b>5'-UTR</b>	transcribed but not translated region of a (-) <b>gene</b> , upstream of a (-) <b>coding region</b> ; in (-) <b>plastid (-) genes</b> , the <b>5'-UTR</b> contains sequence information for translation initiation (ribosome binding site, (-) <b>RBS</b> ) close to its 3' end
<b>aadA</b>	(-) <b>coding region</b> of bacterial aminoglycoside adeny transferase, a frequently used protein, that detoxifies antibiotic (-) <b>selection inhibitors</b> spectinomycin and/or streptomycin
<b>chloroplast</b>	(-) <b>plastid</b> containing chlorophyll
<b>coding region</b>	nucleotide sequence containing the information for a) the amino acid sequence of a polypeptide or b) the nucleotides of a functional RNA; coding regions are optionally interrupted by one or more (-) <b>intron(s)</b>
<b>desired gene, (sequence)</b>	modified or newly introduced sequence: the purpose of a (-) <b>transformation attempt</b>
<b>flank, flanking region</b>	DNA sequences at the 5' and 3' ends of inserts in a (-) <b>plastid (-) transformation (-) vector</b> , which mediate integration into the target (-) <b>plastome</b> of sequences between the flanks by double reciprocal (-) <b>homologous recombination</b> . By the same mechanism, sequences can be modified or removed from the target (-) <b>plastome</b> . Thus, the flanks of the (-) <b>plastid (-) transformation (-) vector</b> determine, where changes in the target (-) <b>plastome</b> are generated by (-) <b>transformation</b> .

<b>gene expression</b>	process, turning sequence information into function; in (→) <b>genes</b> encoding polypeptides, gene expression requires the activity of a (→) <b>promoter</b> which initiates and directs RNA polymerase activity, leading to the formation of a messenger RNA, which is subsequently translated into a polypeptide; in (→) <b>genes</b> encoding RNA, the (→) <b>promoter</b> -mediated activity of RNA polymerase generates the encoded RNA
<b>gene(s)</b>	nucleotide sequence(s) encoding all elements, which are required to secure function independently; genes are organised in (→) <b>operons</b> , which contain at least one complete (→) <b>coding region</b> in (→) <b>genes</b> encoding polypeptides, these elements are: (1) a (→) <b>promoter</b> , (2) a 5' untranslated region ((→) <b>5'-UTR</b> ), (3) a complete (→) <b>coding region</b> , (4) a 3' untranslated region ((→) <b>3'-UTR</b> ); in (→) <b>genes</b> encoding RNA, the (→) <b>5'-UTR</b> and the (→) <b>3'-UTR</b> are missing; in (→) <b>operons</b> consisting of more than one (→) <b>coding region</b> , two subsequent complete (→) <b>coding regions</b> are separated by a (→) <b>spacer</b> , and (→) <b>promoter</b> , (→) <b>5'-UTR</b> , and (→) <b>3'-UTR</b> elements are shared by the (→) <b>coding regions</b> of that (→) <b>operon</b> .
<b>genome</b>	Complete DNA sequence of a cell's nucleus or a cell organelle
<b>hcf</b>	high chlorophyll fluorescence; hcf mutants show a characteristic photosynthesis deficient phenotype
<b>heteroplastomic</b>	
<b>plastid / cell</b>	a (→) <b>plastid</b> or cell containing genetically different plastomes
<b>homologous</b>	
<b>recombination</b>	process leading to exchange, insertion or deletion of sequences due to the presence of (→) <b>flanks</b> with sufficient sequence homology to a target site in a (→) <b>genome</b>
<b>homoplastomic</b>	
<b>plastid / cell</b>	a (→) <b>plastid</b> or cell containing genetically different plastomes
<b>insertion site</b>	locus in the (→) <b>plastome</b> , into which novel sequences are introduced



<b>Intergenic region</b>	sequences between two (→) <b>genes</b> in a (→) <b>genome</b> ; such region occur as (→) <b>interoperonic regions</b> or as (→) <b>intraoperonic regions</b> , in which case they are also called (→) <b>spacers</b>
<b>intragenic region</b>	sequences inside a (→) <b>gene</b>
<b>intron</b>	sequence interrupting a (→) <b>coding region</b>
<b>organ</b>	a plant <b>organ</b> is a structure that serves a special biological function and consists of one or more characteristic (→) <b>tissues</b> ; examples are: root, stem, leaf, flower, stamen, ovary, fruit etc.
<b>operon</b>	organisational structure of (→) <b>genes</b>
<b>petA</b>	(→) <b>coding region</b> of the (→) <b>plastid</b> (→) <b>gene</b> for the cytochrome f protein involved in photosynthetic electron transport
<b>plant(s)</b>	organism(s) that contain(s) (→) <b>plastids</b> in its cells; this invention relates to multicellular (→) <b>plants</b> ; these include the group of gymnosperms (such as pine, spruce and fir etc.) and angiosperms (such as <i>monocotyledonous</i> crops e.g. maize, wheat, barley, rice, rye, Triticale, sorghum, sugar cane, asparagus, garlic, palm tress etc., and non-crop monocots, and <i>dicotyledonous</i> crops e.g. tobacco, potato, tomato, rape seed, sugar beet, squash, cucumber, melon, pepper, Citrus species, egg plant, grapes, sunflower, soybean, alfalfa, cotton etc.), and non-crop dicots as well as ferns, liverworts, mosses, and multicellular green, red and brown algae.
<b>plastid(s)</b>	organelle(s) with their own genetic machinery in (→) <b>plant</b> cells, occurring in various functionally and morphologically different forms, e.g. amyloplasts, (→) <b>chloroplasts</b> , chromoplasts, etioplasts, gerontoplasts leukoplasts, proplastids etc.
<b>plastome</b>	complete DNA sequence of the (→) <b>plastid</b>
<b>promoter</b> <b>RBS,</b> <b>ribosomal binding</b> <b>site</b>	nucleotide sequence functional in initiating and regulating transcription   DNA sequence element upstream of the (→) <b>translation start codon</b> of a (→) <b>coding region</b> , that mediates ribosome binding and translation

initiation from the respective RNA transcript; RBS elements are either part of (→) 5'-UTRs or of **spacers**.

***rpoA/B/C***

(→) **coding regions** of the (→) **plastid (→) genes** for the plastid encoded RNA-Polymerase (PEP)

**selection inhibitor**

chemical compound, that reduces growth and/or development of non-transformed cells or organelles stronger than that of transformed ones

**tissue**

a **plant tissue** consists of a number of cells with similar or identical structure and function; cells in plant tissues are connected by plasmodesmata; examples are: callus, palisade parenchyma, spongy parenchyma, cambium, epidermis, pith, endosperm, phloem, xylem etc.

**transformation**

**vector**

cloned DNA molecule that was generated to mediate (→) **transformation** of a (→) **genome**;

**transformation**

process leading to the introduction, the excision or the modification of DNA sequences by treatment of (→) **plants** or plant cells including the use of at least one (→) **transformation vector**

**transgene**

DNA sequence derived from one (→) **genome**, introduced into another one

**translation start**

**codon**

sequence element, that encodes the first amino acid of a polypeptide

**translation stop**

**codon**

sequence element that causes discontinuation of translation

***uidA***

(→) **coding region** of bacterial  $\beta$  glucuronidase, a frequently used **reporter protein**

***ycf3***

(→) **coding region** for a protein that is involved in PSI assembly;  $\Delta ycf3$  lines display a pale phenotyp and growth depression, when cultivated under standard light conditions (3.5-4 W/m<sup>2</sup>). Under low light conditions (0.4-0.5 W/m<sup>2</sup>) the phenotype is much less severe.

#### SHORT DESCRIPTION OF THE FIGURES

- Fig. 1: Schematic view of vector pIC571.  
Fig. 2: Schematic view of vector pIC554.  
Fig. 3: Schematic view of vector pGEM-rpoA-del and targeted plastome region.  
Fig. 4: Schematic view of vector pIC598 and targeted plastome region.  
Fig. 5: Schematic view of vector pIC553.  
Fig. 6: Schematic view of vector pKCZ-GFP.  
Fig. 7: Schematic view of vector pIC526.  
Fig. 8: Schematic view of vector pIC558 and targeted plastome region.  
Fig. 9: Schematic view of vector pIC558.  
Fig. 10: Schematic view of vectors pIC597, pIC599 and pIC600 and targeted plastome region.  
Fig. 11: Schematic view of vector pIC597.  
Fig. 12: Map vector pIC577.  
Fig. 13: Schematic view of vector pIC577 and targeted plastome region.  
Fig. 14: Map of vector pIC637.  
Fig. 15: Schematic view of vector pIC637 and targeted plastome region.

#### DETAILED DESCRIPTION OF THE INVENTION

##### *Vectors of this invention provide a visible marker during selection*

Non-lethal inhibitor concentrations that do not kill plant cells but inhibit growth to a certain degree can be used for plastid transformation. Only one or a few of the up to 10.000 plastome copies per cell can be assumed to be recombinant after the initial transformation event. Treating the cultured cells or tissues with a lethal inhibitor concentration after transformation would not allow to recover heteroplastomic cells expressing a moderate resistance, which is due to a low number of transformed plastome copies. Selection and segregation leads to the occurrence of both wild-type and transplastomic tissues. It is a major problem to discriminate between wild-type and transgenic tissue during this process, because transformed plastids may mask wild type ones. Khan and Maliga (1999) used a fluorescent antibiotic resistance marker, comprising the *aadA* and GFP coding regions, to track segregation in plastid transformants under UV-light. In this invention, we present a visible marker for the transplastomic tissue sectors which can be detected by the naked eye. The

gradual process of sorting out wild-type and recombinant plastids can easily be monitored and thus be accelerated. The appearance of green sectors on the white background of the mutant phenotype can be easily detected.

*Vectors of this invention provide improved regeneration efficiency*

Conventional chloroplast transformation strategies are based on the selection for resistance against an inhibitor, e.g. spectinomycin. Inhibitor application starts after transformation and is perpetuated during the whole process of repetitive regenerations which are necessary to obtain a homoplastomic genotype. Inhibitor application has the disadvantage to reduce the regeneration potential. Regeneration of whole plants from single protoplasts or leaf pieces is a critical step in chloroplast transformation, particularly when extending the method to species, for which established and reliable regeneration protocols do not exist. It is a major advantage of this invention, that inhibitors may only have to be utilized during a short period after the first transformation. Using our novel methods, inhibitor application can be omitted during repetitive regeneration in order to achieve a homoplastomic condition and during the complete second transformation step.

*Vectors of this invention allow generation of genetically stable plastome transformants*

Homologous recombination in plastids is known to occur with high efficiency. As a consequence, undesired recombination events between regulatory elements of an antibiotic resistance marker and endogenous regulatory elements may lead to genetic instability (Eibl et al., 1999). After the second transformation step the transplastomic plant does not contain any marker expression cassette. Consequently the final transformants contain fewer regions of homology than conventional plastid transformants. The genetic stability of the transformants is increased and undesired loss of sequences (Eibl et al., 1999) is avoided.

By this invention, a novel antibiotic-free, photosynthesis related selection system for chloroplast transformation of higher plants can be provided. The new system utilizes visible markers and may be based on the inactivation of genes like *rpoA*, *rpoB*, *ycf3* or *petA* yielding a white or pale phenotype. In a second step, the respective deficient gene of the mutant line may be restored and one or more transgenes may be inserted. The resulting transplastomic plants may be free of an antibiotic resistance gene.

Other possible target functions for step (a) of the process of this invention are any plastid encoded functions which are directly or indirectly required for photosynthesis. Apart

from the specific applications described below, inactivation and restoration of numerous photosynthesis related target genes, such as e.g. *psbA* may be used according to this invention.

Plastid chromosomes encode four RNA polymerase genes, designated *rpoA*, *B*, *C1* and *C2*, that resemble the three RNA polymerase core genes of eubacteria. The genes for *rpoB*, *C1* and *C2* are arranged in an operon (transcribed by NEP, a nuclear encoded plastid RNA polymerase), while the gene for *rpoA* is located in a large cluster of genes that mainly encode ribosomal proteins. Since the level of the sense transcript of the *rpoA* gene decreases in PEP (plastid encoded plastid RNA polymerase) deficient mutants (Krause *et al.*, 2000), *rpoA* might be transcribed by PEP.

Deletion of *rpoA*, *rpoB* or *rpoC1* from the plastid genome results in a pigment-deficient phenotype (Allison *et al.*, 1996; De Santis-Maciossek *et al.*, 1999). The pigment-deficient  $\Delta rpoA$ ,  $\Delta rpoB$  or  $\Delta rpoC1$  plants (white plants) are unable to grow photoautotrophically. However, if maintained on sucrose-containing medium to compensate for the lack of photosynthesis, they grow normally but at a reduced rate compared with wild-type plants.

*ycf3* has recently been shown to be required for stable accumulation of the photosystem I (PSI) complex in tobacco (Ruf *et al.*, 1997). Disruption of this gene leads to a conditional pigment-deficient phenotype in light. Homoplasmic *ycf3* plants display a completely white phenotype upon regeneration on drug- and phytohormone-free medium under standard light conditions (3.5–4 W/m<sup>2</sup>), while the phenotype is much less severe (light green) under low light conditions (0.4–0.5 W/m<sup>2</sup>).

A mutant plant phenotype which is called hcf (high chlorophyll fluorescence) is well known. This phenotype is due to a mutation in expression and/or processing of photosynthesis related genes (either nuclear or plastome encoded genes; Bock *et al.* 1994; Monde *et al.*, 2000; Monde *et al.*, 2000b). These mutants show a characteristic photosynthesis deficient phenotype: impaired growth under greenhouse conditions, pale green leaves, and high chlorophyll fluorescence (red fluorescence) under UV-light illumination. Hcf appears when the photosynthetic electron transport chain is blocked ('electron tailback'). One possibility to achieve a hcf phenotype is to inactivate the plastid *petA* gene, which codes for a subunit of the cytochrome b/f complex involved in photosynthetic electron transport.

Taking advantage of the pigment or photosynthesis deficient phenotypes of, for example,  $\Delta rpo$ ,  $\Delta ycf3$  or  $\Delta petA$  plants, a second round of transformation may be performed

using the first round pigment-deficient transformants as a substrate to restore the deficiency, remove the selection marker of the first round, if any, and optionally introduce sequences of interest simultaneously. Green plants can be recovered by delivering a wild type gene into the plastome of pigment-deficient mutants. Therefore, such secondary transformants will regain the ability of photosynthetic growth and display a green phenotype and/or normal chlorophyll fluorescence in case of the *hcf* phenotype. This characteristic can be used to select transformed tissues. Thus no antibiotic selection is required in this second step. More importantly, selection markers used during the first round of transformation can be removed in the second round, yielding marker-free transplastomic plants.

Plastid gene transformation is based on homologous recombination. This can be achieved by using, in a transformation vector, flanking regions of sufficient homology to target sites on the plastome, which is well-known in the art. In the present invention, transformation may be performed by any method known in the art. Presently, there are two such known methods, namely particle gun transformation and PEG-mediated transformation. In this invention, particle gun transformation is preferred. Both steps (a) and (c) of the process of this invention may also be achieved by co-transformation, i.e. using more than one transformation vector.

The teaching of this invention may be applied to any multicellular plant. Preferred plants are monocot and dicot crop plants. Specific examples of crop plants are listed herein under item "plants" in section "Definitions".

There are several possibilities for altering or disrupting the function of a plastid gene in step (a) provided that a selectable or recognizable phenotype is produced. Examples include partial or full deletion of the coding region of said gene or of a functional element required for expression of said gene e.g. promoter, 5'-UTR, 3'-UTR, and start codon. The function of these elements may also be altered or disrupted by insertion of a foreign sequence into these elements or the coding region, by full or partial replacement of these elements by a foreign sequence or by insertion of a stop codon into the coding region. The above means may also be combined. If a resistance marker gene is introduced in step (a), the marker gene is preferably used as said foreign sequence. In step (a) any additional sequence of interest may be inserted concomitantly.

Step (a) of the process of this invention is preferably achieved by genetic transformation. In an alternative embodiment, step (a) may occur or may have occurred by a spontaneous or induced mutation. This means that a plant (or plant organs or plant tissue)

used for step (c) of this invention may be a natural mutant or a transgenic plant not obtained according to this invention or for purposes of this invention, which is predominantly the production of transgenic plants free of a marker gene.

In step (b) of the process of this invention, plants, plant organs or plant tissue having plastids expressing the phenotype of interest are separated or selected for on medium supporting heterotrophic growth. Selection may be done using a selectable marker gene introduced in step (a) and a suitable antibiotic or inhibitor. Alternatively, the novel procedures of this invention using photosystem I acceptor herbicides as described in more detail below may be applied. In the latter case, no resistance gene has to be introduced in step (a). As described above, inhibitors or antibiotics do only have to be utilized during a short period after the first transformation to support segregation and the use of such an agent can even be neglected totally. After growth and several cell divisions, segregation leads to the formation of zones differing in pigment abundance or fluorescence. Tissue from such zones is separated manually and is used for further rounds of regeneration.

In step (c) of the process of this invention, the plastome of a plant obtained in previous steps is transformed with a vector having a restoring sequence capable of restoring the function altered or disrupted in step (a). Said restoring may be achieved by several means dependent on how alteration or disruption in step (a) was done. Inserted sequences may be removed, replaced sequences may be replaced again with the original fully functional sequence, and deleted sequences may be reinserted. Concomitantly, a resistance gene inserted in step (a) may be removed or its function may be destroyed and an additional genetic modification of the plastome may be carried out or an additional function may be introduced. Examples include introduction of an additional sequence or gene of interest, introduction of several genes, elimination of a preexisting function or sequence etc.

In step (d), plants, plant organs or plant tissue having plastids expressing said restored function are separated or selected for on antibiotic-free medium. Selection may be achieved by at least partial phototrophic growth and transformed plants are recognizable by the restored phenotype. Upon growth, segregation leads to the formation of zones of differing pigment abundance. Green zones are separated manually and used for further rounds of regeneration. In this invention, the conditions used in step (d) are preferably mixotrophic. This means that the carbohydrate content of the medium is lowered as much as possible such that plastids containing transformed plastomes and cells containing transformed plastids which have regained the ability for photosynthesis have a selective growth advantage under strong light. Such mixotrophic conditions may accelerate step (d).

*Embodiment 1: Method for the selection of plastid transformants based on the inactivation and restoration of the rpoA or rpoB genes*

For targeted disruption of *rpoB* gene function, the *rpoB* promoter and the start codon may for example be replaced with the *aadA* marker gene or another marker gene. Bombarded leaf tissue may be regenerated under temporary selection on spectinomycin-containing medium in case of the *aadA* marker. Transformants display antibiotic resistance and initially a green phenotype in light while still being heteroplastomic. These primary transformants contain a mixture of both wild-type and transformed chloroplast genomes. The green, heteroplastomic material is transferred to non-selective medium. Segregation leads to the occurrence of white, mixed, and green sectors. Material from white sectors may be subjected to several additional rounds of regeneration on non-selective medium in order to obtain homoplastomic mutant transformants.

In the second transformation, the homoplastomic  $\Delta rpoB$  plants may be transformed with a vector designed to reconstitute the *rpoB* gene, remove the marker gene and preferably introduce a gene (or genes) of interest at the same time. The treated leaf tissue may be regenerated under selection on sucrose-reduced medium (antibiotic free) under strong light. Transformants which display a green phenotype and are able to grow photoautotrophically may be selected.

A disruption and reactivation of the *rpoA* gene may be achieved in a similar way.

*Embodiment 2: Method for the selection of plastid transformants based on the inactivation and restoration of the ycf3 gene*

Disruption of *ycf3* gene may be achieved by replacing the 5'-regulatory element and the first exon of *ycf3* by a marker gene like the *aadA* marker gene. The transformation vector may be introduced into tobacco plastids e.g. using the biolistic protocol or PEG-mediated transformation. The bombarded leaf tissue, in case of the biolistic protocol, is regenerated under selection on medium containing an inhibitor or antibiotic, spectinomycin in case of the *aadA* gene. Transformants display inhibitor resistance and initially a green phenotype under standard light conditions (3.5-4 W/m<sup>2</sup>) while still being heteroplastomic. These primary transformants normally contain a mixture of wild-type and transformed chloroplast genomes. After transfer to antibiotic-free medium, segregation leads to the occurrence of yellow-white and green sectors (under standard light conditions; see above). Material from white sectors



may be subjected to several additional rounds of regeneration on non-selective medium in order to obtain homoplastomic mutant transformants. Besides having a pale, nearly white phenotype in light, the mutants show depressed growth. To obtain adequate material for the second transformation step, the mutant plant line may be transferred to low light conditions. Under these conditions (0,4-0,5 W/m<sup>2</sup>), the plants show a much less severe phenotype and can yield suitable donor material e.g. for particle gun transformation. In this second transformation, the homoplastomic  $\Delta ycf3$  plants are transformed with a vector designed to reconstitute the *ycf3* gene, remove the marker gene and preferably introduce a gene (or genes) of interest at the same time. The bombarded leaf tissue may be regenerated under selection on sucrose-reduced medium (antibiotic free) under strong light. Transformants, which display a normal green phenotype and are able to grow photoautotrophically, may be selected.

*Embodiment 3: Method for the selection of plastid transformants based on the inactivation and restoration of the *petA* gene*

For targeted disruption of the *petA* gene, the coding region may be replaced with a marker gene, e.g. the *aadA* marker gene. Bombarded leaf tissue, in the case of transformation by particle bombardment, may be regenerated under selection on antibiotic containing medium. Transformants display antibiotic resistance and initially a normal green phenotype in light while still being heteroplastomic. These primary transformants contain a mixture of wild-type and transformed chloroplast genomes. After transfer to antibiotic-free medium, segregation may lead to the occurrence of sectors displaying the *hcf* phenotype, which can be detected under UV illumination. Material from the mutant sectors may be subjected to several additional rounds of regeneration on non-selective medium in order to obtain homoplastomic mutant material.

In the second transformation, the homoplastomic  $\Delta petA$  plants may be transformed by bombardment with a transformation vector designed to reconstitute the  $\Delta petA$  gene, remove the marker gene and preferably introduce a gene (or genes) of interest at the same time. The bombarded leaf tissue may be regenerated under selection on sucrose-reduced medium (antibiotic free) under strong light. Transformants which display a normal green phenotype and are able to grow photoautotrophically may be selected.

*Embodiment 4: Method for the selection of plastid transformants based on the inactivation and restoration of the *petA* gene, whereby inactivation mutants are selected by a novel procedure*

This procedure may be used with all mutants that are photosynthesis defective. Similar to embodiment 3, selection of plastid transformants may be based on the inactivation and restoration of the *petA* gene. In contrast, the selection for  $\Delta petA$  mutants may be carried out on medium containing a herbicide that requires active photosynthesis for efficacy, e.g. the herbicide Paraquat. Any complete inactivation of the *petA* gene may result in an increased resistance of the mutant plant line to such a herbicide compared to wild-type. Importantly, the introduction of any antibiotic or herbicide resistance marker during the first transformation step can be omitted.

*Vectors of this invention provide the possibility to introduce novel functions during the first or the second transformation step*

Genes or sequences of interest may be introduced during the first or the second step of transformation or during both. Therefore, multiple genes or functional operons may be introduced into the target plant. Among others, this is of particular interest for the generation of new metabolic pathways in transplastomic plants, as a significant number of novel genes and/or regulation factors may have to be integrated into the plastome.

Similarly, desired sequences may be introduced or removed e.g. in order to manipulate plastid gene expression pattern.

*Vectors of this invention provide the possibility to reuse selection markers*

A further application of the two step strategy described in this invention is the possibility to reuse the same selection marker for another transformation after removing it from the genome during the second step. Subsequently, it may be removed again and the process may be repeated. This provides means for the insertion of a potentially unlimited number of genes or functional operons into the plastid genome using the same marker gene. This is an important step towards overcoming the shortage of selection markers for plastid gene transformation.

*Vectors of this invention allow the introduction of sequences of interest at independent loci*

By using various combinations of the described methods (e.g. embodiment 1, 2 and 3 using *rpoA*, *ycf3* and *petA*, respectively, as target sites), the genes of interest can be introduced into different target sites of the plastome. The method described here will also

function using inactivation and restoration of other photosynthesis related target genes, such as e.g. *psbA*. Consequently numerous potential target sites exist. Using homologous recombination, novel functions may also be introduced at sites independent of marker genes.

*Vectors of this invention provide novel selection schemes*

The *aadA* gene is the only selectable marker gene that is used routinely (Heifetz, 2000), and the *nptII* gene conferring resistance to kanamycin is the only alternative that has been shown to function in higher plant plastid transformation (Carrer et al., 1993). The vectors of this invention overcome the shortage of selectable marker genes. Novel selection inhibitors for plastid transformation that are described here include paraquat, morphamquat, diquat, difenzoquat and cyperquat. These substances belong to the group of photosystem I acceptor herbicides (Hock & Elsner, 1995). They are not inhibitors of photosystem I, but they are reduced by photosystem I instead of ferredoxin and NADP. Autooxidation of reduced inhibitors then produces oxygen radicals which are highly toxic. The toxicity of these herbicides therefore depends on light and oxygen.

If the electron transport through photosystem I is interrupted either by deletion of an essential gene of photosystem I or of the cytochrome b/f complex (for example *petA*) these mutant plants are more resistant to photosystem I acceptor herbicides like paraquat than wild-type plants. Such herbicides may therefore be suitable selection agents for the knock out of these genes. Any albino will be insensitive to these inhibitors; thus any disruption that leads to photosynthesis deficiency could be selected for this way.

In one embodiment of the invention, step (d) may be assisted by inhibitor resistance. Inhibitor resistance may be achieved via stable or transient introduction of an inhibitor or antibiotic resistance in step (c). Preferably, an inhibitor resistance introduced in step (c) is transient in order to allow the generation of selection marker free transplastomic plants as an end result. An inhibitor resistance gene may be removed by methods known in the art, e.g. as described by Fischer et al. (1996) or by Lamtham and Day (2000). Further, an inhibitor or antibiotic may be applied only initially in step (d) similarly as described above for step (b). Omitting the inhibitor at a later stage allows loss of a resistance gene introduced in step (c). This embodiment takes advantage of the regeneration of a discernible phenotype in steps (c) and (d) according to the principles of the invention, but the achievement of a homoplasmic state in step (d) may be made more efficient.

## EXAMPLES

The invention will be further described by reference to the following detailed examples. These examples are provided for purposes of illustration only, and are not intended to be limiting unless otherwise specified. Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Ausubel et al., 1999, Maniatis et al., 1989 and Silhavy et al., 1984.

### Example 1: Construction of a selection system based on the inactivation of the *rpoB* gene

#### *Construction of transformation vector pIC571 for the inactivation of the rpoB gene*

Leaves of tobacco plants were ground under liquid nitrogen and total DNA (*Nicotiana tabacum* L. var. petit havanna) was isolated using the Qiagen "DNeasy Plant Mini Kit".

Using this total genomic DNA as a template the region of the tobacco chloroplast genome containing the *rpoB* and *trnA7* genes were amplified by PCR. The following pair of oligonucleotide primers was used: p38 5'-AAG ATG AAC CTG TTC CCA TG -3' (annealing with plastome nucleotides 25967-25986; position numbers according to gene bank accession number Z00044.1) and p39 5'-CAC TTC TTC CCC ACA CTA CG-3' (annealing with plastome nucleotides 29616-29597). The PCR amplification using Taq-polymerase (Sigma) was performed as follows: 60 sec at 95°C, 1 cycle; 30 sec at 94°C, 60 sec at 55°C, 240 sec at 72°C, 32 cycles; final extension at 72°C for 10 min. The reaction products were analysed by agarose gel electrophoresis. Only a single fragment could be detected. It showed the expected size of 3.65 kbp. The fragment was ligated into vector pCRII (Invitrogen) according to the protocol of the supplier, yielding plasmid pCR *rpoB*01. The identity of the plasmid insert was verified by sequencing (Toplab; Munich).

To inactivate the plastid *rpoB*-Operon, a selectable *aadA* marker cassette should replace the 5'-upstream region and the translation start of *rpoB*, represented by a 699 bp *Ava* I fragment (plastome position 27508-28206). As a prerequisite, an additional *Ava* I restriction site in the multiple cloning site of plasmid pCR *rpoB*01 had to be removed. This was done by cutting the plasmid with the enzyme *Xho* I, followed by a fill-in reaction using Klenow polymerase and nucleotides. The linear fragment was then religated and transformed into bacteria. As a consequence the resulting plasmid pCR *rpoB* Δ*Xho* only contained the two *Ava* I sites mentioned above.

Plasmid pCR *rpoB* Δ*Xho* was digested with *Ava* I. The larger of the two resulting

fragments (6861 bp and 699 bp) was purified from an agarose gel using the Qiagen gel extraction kit. The resulting sticky ends of the 6861 bp fragment created by the *Ava* I treatment were converted into blunt ends using Klenow enzyme and nucleotides. The resulting DNA was treated with calf alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany) to suppress self ligation in the following step.

Finally, a 1412 bp *Sma* I fragment containing the *aadA* expression cassette from vector pUC16SaadA-*Sma* (Koop et al., 1996) was ligated into the 6861 bp *Ava* I fragment. The ligation products were transformed into bacteria. The plasmids of the resulting bacterial clones were analyzed for the presence and the orientation of the *aadA*-insert. 7 positive clones showed insertion of the *aadA*-cassette in sense direction compared to the *rpoB* gene. The plasmid was designated pIC571 (pCR *rpoB aadA*-I) (Fig. 1). Large amounts of pCR *rpoB aadA*-I plasmid DNA were isolated using the Qiagen Plasmid Maxiprep kit.

#### *Primary transformation and selection of homoplastomic $\Delta rpoB$ mutants*

PEG mediated transmembrane DNA transfer into protoplasts is a reproducible method for plastid transformation of higher plants (Golds et al., 1993; O'Neill et al., 1993). Protoplast regeneration was recently optimized according to Dovzhenko et al., 1998.

**A. Protoplast isolation:** Leaves from about 3 weeks old tobacco plants (*Nicotiana tabacum* cv. petit Havanna) were cut to 1 mm stripes and incubated overnight with 0,25 % cellulase R10 and 0,25 % macerozyme R10 (Yakult, Honsha Japan) dissolved in F-PIN medium. Following standard filtration, flotation and sedimentation procedures (Koop et al., 1996) protoplasts were resuspended in transformation medium, the total number of protoplasts was determined, and the density was adjusted to  $5 \times 10^6$  protoplasts per ml.

F-PIN medium (pH 5,8 (KOH), osmolarity: 550 mOsm):  $\text{KNO}_3$  (1012  $\mu\text{g/ml}$ ),  $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$  (440  $\mu\text{g/ml}$ ),  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$  (370  $\mu\text{g/ml}$ ),  $\text{KH}_2\text{PO}_4$  (170  $\mu\text{g/ml}$ ),  $\text{NH}_4$ -succinate (10 ml of 2M stock), EDTA-Fe(III) · Na-salt (40  $\mu\text{g/ml}$ ), KJ (0,75  $\mu\text{g/ml}$ ),  $\text{H}_3\text{BO}_3$  (3  $\mu\text{g/ml}$ ),  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (10  $\mu\text{g/ml}$ ),  $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$  (2  $\mu\text{g/ml}$ ),  $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$  (0,25  $\mu\text{g/ml}$ ),  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$  (0,025  $\mu\text{g/ml}$ ),  $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$  (0,025  $\mu\text{g/ml}$ ), inositol (200  $\mu\text{g/ml}$ ), pyridoxin-HCl (2  $\mu\text{g/ml}$ ), thiamin-HCl (1  $\mu\text{g/ml}$ ), biotin (0,02  $\mu\text{g/ml}$ ), nicotinic acid (2  $\mu\text{g/ml}$ ), BAP (1  $\mu\text{g/ml}$ ), NAA (0,1  $\mu\text{g/ml}$ ), Polypuffer 74 (10 ml), sucrose (~130 000  $\mu\text{g/ml}$ ).

Transformation medium (pH 5,8 (KOH), osmolarity: 550 mOsm):  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (3050  $\mu\text{g/ml}$ ),

MES (1000 µg/ml), mannitol (~80000 µg/ml).

**B. Plastid transformation and protoplast embedding:** 50 µg DNA (transformation vector pIC571), 7 µl F-PCN, and 100 µl (500.000 cells) of protoplast suspension were added to 125 µl 40 % PEG solution, mixed carefully and incubated for 7.5 min. Another 125 µl of F-PCN were added, mixed and incubated for 2 min. The volume was filled up to 3 ml (with F-PCN) and 3 ml of F-alginate medium was added. Alginate embedding in thin layers is performed by applying 625 µl of protoplast-alginate mixture to polypropylene grids laying on the surface of  $\text{Ca}^{2+}$ -medium. After solidification grids were removed and placed upside down into liquid F-PCN medium for equilibration (2 x 10 ml, 30 min each) and then transferred to a new petri dish with 2 ml F-PCN. The embedded protoplasts were incubated in the darkness for the initial 20 hours, followed by a usual 16 h day/8 h dark cycle.

F-PCN medium (pH 5,8 (KOH), osmolarity: 550 mOsm):  $\text{KNO}_3$  (1012 µg/ml),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (440 µg/ml),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (370 µg/ml),  $\text{KH}_2\text{PO}_4$  (170 µg/ml),  $\text{NH}_4$ -succinate (10 ml of 2M stock), EDTA-Fe(III) · Na-salt (40 µg/ml), KJ (0,75 µg/ml),  $\text{H}_3\text{BO}_3$  (3 µg/ml),  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (10 µg/ml),  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  (2 µg/ml),  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  (0,25 µg/ml),  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  (0,025 µg/ml),  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (0,025 µg/ml), inositol (200 µg/ml), pyridoxin-HCl (2 µg/ml); thiamin-HCl (1 µg/ml), biotin (0,02 µg/ml), nicotinic acid (2 µg/ml), BAP (1 µg/ml), NAA (0,1 µg/ml), Polypuffer 74 (10 ml), sucrose (~20 000 µg/ml), glucose (65 000 µg/ml).

F-alginate medium (pH 5,8 (KOH), osmolarity: 550 mOsm): MES (1370 µg/ml),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (2500 µg/ml),  $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$  (2040 µg/ml), mannitol (~77000 µg/ml), alginate (24000 µg/ml).

$\text{Ca}^{2+}$ -medium (pH 5,8 (KOH), osmolarity: 550 mOsm): MES (1950 µg/ml),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (2940 µg/ml), mannitol (~82000 µg/ml), agar, purified (10000 µg/ml).

One week after transformation embedded protoplasts were transferred to solid RMOP medium (see example 3) containing 500 µg/ml spectinomycin and streptomycin each. Every 3 weeks the grids were transferred to fresh medium until no further regenerates appeared. First green regenerates appeared after 5 weeks and were transferred to single petri dishes. As expected, primary  $\Delta\rho\text{oB}$  transformants displayed spectinomycin-resistance and a green phenotype in the light while still being heteroplastomic. In order to amplify transformed plastid DNA molecules and to eliminate wild-type genomes, the transformant colonies were

transferred to RMOP medium without inhibitors. White sectors appeared after 3 to 5 weeks of culture. Material from white sectors was further subcultured on non-selective medium and subjected to 5 further cycles of regeneration in order to obtain homoplastomic mutant transformants. The resulting lines showed a white phenotype. The transplastomic lines were rooted and propagated on solid VBW-medium (see examples) to obtain mutant plant material for the secondary transformation.

#### *Analysis by PCR and Southern blotting*

Leaves of the mutant  $\Delta rpoB$  transplastomic plants were ground under liquid nitrogen and total DNA (*Nicotiana tabacum* L. var. petit havanna) was isolated using the Qiagen "DNeasy Plant Mini Kit".

Plastid transformants were analyzed by PCR amplification. Total DNA isolated from regenerates of several independent lines were used as templates for separate PCR reactions. Two sets of oligonucleotide primers were used to analyze the transplastomic plants. oFCH59 5'-TGC TGG CCG TAC ATT TGT ACG-3' (derived from the 5' portion of the *aadA* coding region) and oFCH60 5'-CAC TAC ATT TCG CTC ATC GCC-3' (derived from the 3' portion of the *aadA* coding region) were used to detect the presence of the *aadA* gene. p42 5'-ATT TGT AGT AGA AGG TAA TTG C-3' (annealing with plastome nucleotides 29081-29102) and oFCH60 were used to detect correct integration of the *aadA* gene.

Additional proof of correct integration and the homoplastomic genotype was given by DNA gel blot analysis. Genomic DNAs isolated from sterile grown plants were used for DNA gel blot analysis. The detailed procedure was as follows: 3  $\mu$ g of total plant DNA per analyzed plant were digested with the appropriate restriction enzyme and separated on a TAE agarose gel (0.8%). The DNA was denatured and transferred onto a positively charged nylon membrane (Hybond-N+, Amersham) as described in Ausubel et al. (1999). The filter was hybridized with digoxigenin-labeled probes in DIG Easy Hyb Buffer (Roche Diagnostics GmbH, Mannheim, Germany), and hybridization signals were detected using the DIG Luminescent Detection Kit (Roche). The membrane was exposed to an X-OMAT LS film at room temperature for 75 minutes. For preparation of a hybridization probe, a 398 bp *Sma* I / *Hind* III fragment was excised from plasmid pCR *rpoB*01, purified from a agarose gel and labeled using the Dig probe labeling kit (Roche).

*Construction of transformation vector pIC554 for reconstitution of the rpoB gene*

For a proof of principle of the selection system, a transformation vector was constructed, which reconstitutes the deletion of the *rpoB* regulatory region and introduces a marker restriction site at the same time. The additional marker restriction site should allow to differentiate between recombinant plastome fragments in the respective area and a potential selection of residual wildtype plastome copies (in case the mutant lines were not completely homoplastomic).

Plasmid pIC571 (pCR *rpoB*01) was cut with *Xma* I. The ends of the linear fragment were converted into a blunt form using Klenow polymerase and nucleotides. The resulting DNA was religated and transformed into bacteria. Plasmids of the bacterial clones were screened for the absence of the *Sma* I restriction site. DNA from the resulting plasmid pIC554 (pCR *rpoB*01- $\Delta$ *Sma*) (Fig. 2) was isolated for plastid transformation.

The *Sma* I restriction site of plasmid pIC571 enables easy one step integration of any foreign gene to be expressed in plastids.

*Plastid transformation of  $\Delta$ rpoB mutant lines and selection of homoplastomic lines*

The goal of the second transformation is to reconstitute the *rpoB* gene's regulatory region (including the translation start), remove the *aadA*-cassette and introduce a marker restriction site at the same time. Young leaves from sterile homoplastomic  $\Delta$ *rpoB* mutants grown on VBW-medium were bombarded with plasmid pIC554 coated gold particles using the Bio-Rad (Hercules, CA, USA) PDS-1000/He Biolistic particle delivery system (for detailed procedure see example 3). Two days after bombardment, leaves were cut into small pieces (ca. 3x3 mm) and transferred to solid sucrose-reduced-RMOP medium (containing 3g/liter sucrose). Every three weeks the leaf pieces were cut again and transferred to fresh medium until no further regenerates appeared. The transformants which display green phenotype and are able to grow photoautotrophically were selected and subjected to several additional rounds of regeneration on sucrose-reduced-RMOP medium to obtain homoplastomic tissue. Homoplastomic transplastomic lines were rooted and propagated on solid B5-medium.

*Molecular analysis of the secondary transplastomic plants*

Total DNA isolated from sterile grown plants recovered from the secondary transformation was used for DNA gel blot analysis.

The detailed procedure was as follows: 3  $\mu$ g of total plant DNA per analyzed plant were



digested with restriction enzymes *Bam* HI and *Sma* I at the same time and separated on a TAE agarose gel (0.8%). The DNA was denatured and transferred onto a positively charged nylon membrane (Hybond-N+, Amersham) as described in Ausubel et al. (1999). The filter was hybridized with digoxigenin-labeled probes in DIG Easy Hyb Buffer (Roche Diagnostics GmbH, Mannheim, Germany), and hybridization signals were detected using the DIG Luminescent Detection Kit (Roche). The membrane was exposed to an X-OMAT LS film at room temperature for 75 minutes.

For preparation of a hybridization probe, a 398 bp *Sma* I / *Hind* III fragment was excised from plasmid pCR rpoB01, purified from an agarose gel and labeled using the Dig probe labeling kit (Roche). This probe should result in a signal of 3629 bp from the secondary transformed plastomes. This is a clear evidence, that the recombinant fragment from the transformation vector has been integrated, as a potentially wild type derived band would have a size of only 1628 bp. The presence of the 3629 bp fragment also indicates the removal of the *aadA* marker cassette.

To confirm the removal of the *aadA* marker a second hybridization of the blot (of which the former probe had been removed by a stripping procedure) was done using a 480 bp fragment of the *aadA*-gene as probe. For probe generation primers oFCH59 and oFCH60 (see above) were used in a PCR Dig labeling reaction according to the protocol of the supplier (Roche).

## **Example 2: Construction of a selection system based on the inactivation of the *rpoA* gene**

### *Construction of transformation vector pGEM-rpoA-del for the inactivation of the rpoA gene*

The region of the tobacco chloroplast genome (corresponding to plastome nucleotides 79401-82470) containing the *rpoA* reading frame was amplified from genomic DNA isolated from leaf tissue of tobacco by PCR using Taq-polymerase (Qiagen). The following pair of oligonucleotide primers was used: p78 5'-*Sph* I-TTA GTA ACA AGC AAA CCT TG-3' (annealing with plastome nucleotides 79401-79420), and p77 5'-*Sma* I-TAA TTA CTG AAT CGC TTC CCA-3' (annealing with plastome nucleotides 82470-82450).

The PCR program used was as follows: 2 min at 94°C, 1 cycle; 45 sec at 94°C, 45 sec at 55°C, 2 min at 72°C, 30 cycles; final extension at 72°C for 10 min. The fragment was ligated into the pGEM-T vector (Promega). The complete *rpoA* coding region (corresponding to plastome nucleotides 80455-81468) was subsequently deleted by digestion with *Dra*I and *Sca*

I. A chimeric *aadA* gene was excised from pUC16SaadA (for a detailed description of pUC16SaadA see Koop et al., 1996) as a *Sma* I fragment and inserted to replace *rpoA* and to facilitate selection of chloroplast transformations. A plasmid clone carrying the *aadA* gene in the opposite orientation as *rpoA* yielded transformation vector pGEM-*rpoA*-del (Fig. 3). The identity of the plasmid insert was verified by sequencing (MWG, Munich).

*Primary transformation and selection of homoplastomic  $\Delta rpoA$  mutants*

Young leaves from sterile tobacco plants (cultivation see example 1) were bombarded with plasmid pGEM-*rpoA*-del coated gold particles using the Bio-Rad (Hercules, CA, USA) PDS-1000/He Biolistic particle delivery system (detailed procedure see example 3). Two days after bombardment, leaves were cut into small pieces (ca. 3x3 mm) and transferred to solid RMOP-medium containing 500µg/ml spectinomycin. Leaf pieces were cut again and transferred to fresh medium after 2 weeks, then every 3 weeks until no further regenerants appeared. Primary  $\Delta rpoA$  transformants displayed spectinomycin-resistance and a green phenotype in the light while still being heteroplastomic. In order to amplify transformed plastid DNA molecules and to eliminate wild-type genomes, the primary transformants were subjected to 3 additional rounds of regeneration on selective medium. Since segregation leads to the occurrence of white and green sectors, material from white sectors was subjected to several additional rounds of regeneration on non-selective medium in order to obtain homoplastomic mutant transformants. Homoplastomic transformed lines were rooted and propagated on solid VBW-medium (Aviv and Galun, 1985; see example 1).

*Molecular analysis of potential plastid transformants by Southern analysis*

3 µg of total plant DNA per analysed plant were digested with the appropriate restriction enzyme and separated on a TBE-agarose gel (0.8%). The DNA was denatured and transferred to a positively charged nylon membrane (Hybond-N+, Amersham) as described in Ausubel et al., 1999. The filter was hybridised with digoxigenin-labeled probes in DIG Easy Hyb Buffer (Roche Diagnostics GmbH, Mannheim, Germany), and hybridisation signals were detected using the DIG Luminescent Detection Kit (Roche). The membrane was exposed to a X-OMAT LS film at room temperature.

A fragment suitable for discrimination between wild type and transformed plastome was gel purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), labelled with digoxigenin using the Roche DIG DNA Labelling Kit and used for hybridisation.

*Construction of the transformation vector for reconstitution of the rpoA gene*

For a demonstration, that any gene of interest may be inserted using the described selection system, a transformation vector was constructed, which reconstitutes the deletion of the *rpoA* coding region and introduces a *gus* marker gene at the same time.

This vector contains the *rpoA* coding region and the *gus* gene, flanked by 5'- and 3'-homologous sequences which were amplified from the tobacco chloroplast genome by PCR using the following two pairs of primers: oFCH112 5'-*Nco* I-TAC TAT TAT TTG ATT AGA TC-3' (annealing with plastome nucleotides 81471-81490), oFCH113 5'-*Sma* I-TAA TTA CTG AAT CGC TTC CCA-3' (annealing with plastome nucleotides 82470-82450), and oFCH114 5'-*Sph* I-TTA GTA ACA AGC AAA CCT TG-3' (annealing with plastome nucleotides 79401-79420), oFCH137 5'-*Pst* I-ATC ACT AGT TGT AGG GAG GGA TCC ATG GTT CGA GAG AAA GTA AC -3' (annealing with plastome nucleotides 81468-81449). The amplified 5'-homologous fragment (corresponding to plastome nucleotides 81471-82470) contains 1000 nucleotides upstream of the *rpoA* start codon. The amplified 3'-homologous fragment (corresponding to plastome nucleotides 79401-81468) contains a ribosome binding site (RBS), the *rpoA* coding region, and 1054 nucleotides downstream of the *rpoA* stop codon. The 5' and 3'-homologous fragments are subcloned into plasmid pUC16SRBSuidA3'rbcl (Köop et al., 1996), regenerating transformation vector pIC598. The construction of this vector is shown in figure 4. The identity of the plasmid insert was verified by sequencing (MWG, Munich).

*Plastid transformation of  $\Delta rpoA$  mutant lines and selection of homoplastomic lines*

The goal of the second transformation is to reconstitute the *rpoA* coding region, remove the *aadA*-cassette and introduce the *gus* marker gene at the same time. Young leaves from sterile homoplastomic  $\Delta rpoA$  mutants grown on VBW-medium were bombarded with plasmid pIC598-coated gold particles using the Bio-Rad (Hercules, CA, USA) PDS-1000/He Biolistic particle delivery system (detailed procedure see example 3). Two days after bombardment, leaves were cut into small pieces (ca. 3x3 mm) and transferred to solid sucrose-reduced-RMOP medium (containing 3g/liter sucrose). Every three weeks the leaf pieces were cut again and transferred to fresh medium until no further regenerates appeared. Transformants which display green phenotype and are able to grow photoautotrophically were selected and subjected to several additional rounds of regeneration on sucrose-reduced-RMOP medium to

obtain homoplastomic tissue. Homoplastomic transplastomic lines were rooted and propagated on solid B5-medium.

*Molecular analysis of potential plastid transformants by Southern analysis*

3 µg of total plant DNA per analysed plant were digested with the appropriate restriction enzyme and separated on a TBE-agarose gel (0.8%). The DNA was denatured and transferred to a positively charged nylon membrane (Hybond-N+, Amersham) as described in Ausubel et al., 1999. The filter was hybridised with digoxigenin-labelled probes in DIG Easy Hyb Buffer (Roche Diagnostics GmbH, Mannheim, Germany), and hybridisation signals were detected using the DIG Luminescent Detection Kit (Roche). The membrane was exposed to an X-OMAT LS film at room temperature.

A fragment suitable for discrimination between wild type and transformed plastome was gel purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), labelled with digoxigenin using the Roche DIG DNA Labelling Kit and used for hybridisation.

**Example 3: Construction of a white/green selection system based on inactivation of the *ycf3* gene**

*Construction of transformation vector pIC553 for targeted inactivation of the *ycf3**

The region of the tobacco chloroplast genome containing the *ycf3* reading frame was amplified from genomic DNA isolated from leaf tissue of tobacco by PCR using *Taq*-polymerase (QIAGEN). The following pair of oligonucleotide primers was used: oFCH63 (5'-GAA GTT TCT TTC TTT GCT ACA GC-3', annealing with plastome nucleotides 45033-45053) and oFCH64 (5'-GAA TTA CCA AAC CAT TTG ACC C-3', annealing with plastome nucleotides 47667-47647).

The PCR program used was as follows: 2 min at 94°C, 1 cycle; 45 sec at 94°C, 45 sec at 55°C, 2 min at 72°C, 30 cycles; final extension at 72°C for 10 min. The fragment was ligated into the pGEM-T vector (Promega), regenerating plasmid pIC517. The first exon and 5' regulatory element of *ycf3* was subsequently deleted by digestion with *Bbr* PI and *Bst* 1107I. *Bst* 1107I cuts 373 nucleotides upstream of the *ycf3* start codon (nucleotide position 46266). The *Bbr* PI site is located within intron 1 of *ycf3* (close to the end of the first exon). A chimeric *aadA* gene was excised from pUC16SaadA (for a detailed description of pUC16SaadA see Koop et al., 1996) as a *Sma* I fragment. It was inserted to replace *ycf3* and to facilitate

selection of plastid transformants. A plasmid clone carrying the *aadA* gene in the opposite orientation as *ycf3* yielded transformation vector pIC553 (Fig. 5). The identity of the plasmid insert was verified by sequencing (MWG, Munich).

#### *Electrotransformation of E. coli cells*

Preparation of electrocompetent cells: 1 liter of LB-medium (1% (w/v) casein hydrolysate, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl) is inoculated 1:100 with fresh overnight culture of *E. coli* JM109 cells (Promega, Madison, WI, USA). The cells are grown at 37°C with shaking at 220 rpm to an optical density of 0.5 at 600 nm. The cells are chilled on ice for 20 min and centrifuged for 15 min (4000 rpm, 4°C). The supernatant is removed and the pellet is resuspended in 1 liter of ice-cold sterile 10% (v/v) glycerol. The cells are centrifuged two times as described before, resuspending the cells in 500 ml and 20 ml of ice-cold sterile 10% (v/v) glycerol, respectively. The cells are centrifuged an additional time and the pellet is resuspended in 2 ml of ice-cold sterile 10% (v/v) glycerol. This suspension is frozen in aliquots of 80 µl and stored at -80°C.

Electrotransformation using the Bio-Rad (Hercules, CA, USA) Micro Pulser electroporation apparatus: The electrocompetent cells are thawed on ice. 40 µl of the cell suspension are mixed with 2 µl of the ligation mixture and transferred into a prechilled, sterile 0.2 cm cuvette (Bio-Rad). The suspension is shaken to the bottom and the cuvette is placed into the chamber slide. The chamber slide is pushed into the chamber and the cells are pulsed at 2.5 kV. The cuvette is removed from the chamber and the cells are suspended in 1 ml of SOC-medium (2% (w/v) casein hydrolysate, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub> and 20 mM glucose). The suspension is shaken for 1 h at 37°C and 100 µl of the suspension is plated on LB plates containing 150 mg/l ampicillin.

#### *Primary transformation and selection of homoplastomic Δycf3 mutants*

Tobacco seeds (*Nicotiana tabacum* cv. *petit havanna*) were surface sterilized (1 min in 70% ethanol, 10 min in 5% Dimanin C, Bayer, Leverkusen, Germany), washed 3 times for 10 min in sterile H<sub>2</sub>O and put on B5 medium (preparation see below). Plants were grown at 25°C in a 16h light/8h dark cycle (0.5 – 1 W/m<sup>2</sup>, Osram L85W/25 Universal-White fluorescent lamps).

6 leaves from 4 weeks old, sterile grown *Nicotiana tabacum* L. var. *petit havanna* plants were cut and transferred on RMOP-medium (preparation see below). 35 µl of a gold suspension (0.6 micron, Biorad, München; 60 mg/ml ethanol) was transferred into a sterile

Eppendorf-cup (Treff, Fisher Scientific, Ingolstadt, Germany), collected by centrifugation and washed with 1 ml sterile H<sub>2</sub>O. The gold pellet was resuspended in 230 µl sterile H<sub>2</sub>O, 250 µl 2.5 M CaCl<sub>2</sub> and 25 µg DNA (transformation vector pIC553) were added. After thoroughly resuspending the mixture, 50 µl 0.1 M spermidin were added, mixed and incubated for 10 min on ice. Then the gold was collected by centrifugation (1 min, 10000 rpm) and washed twice with 600 µl ethanol (100%, p.A.). The gold was collected by centrifugation (1 min, 10000 rpm) and finally resuspended in 72 µl ethanol (100%, p.A.). A macrocarrier was inserted in the macrocarrier holder and 5.4 µl of the gold-suspension were applied. The bombardment was carried out with a Bio-Rad (Hercules, CA, USA) PDS-1000/He Biolistic particle delivery system using the following parameters:

- rupture disc 900 psi
- helium pressure 1100 psi
- vacuum 26-27 inches Hg
- macrocarrier at the top level
- leaf piece at the third level

6 leaf pieces were bombarded each with 5.4 µl gold-suspension. After bombardment the leaf pieces were incubated for 2 days at 25°C on RMOP-medium.

Two days after bombardment, leaves were cut into small pieces (ca. 3x3 mm) and transferred to solid RMOP-medium containing 500µg/ml spectinomycin. Leaf pieces were cut again and transferred to fresh medium after 2 weeks, then every 3 weeks until no further regenerates appeared. Primary *Δycf* transformants displayed spectinomycin-resistance and a green phenotype in the light while still being heteroplastomic. In order to amplify transformed plastid DNA molecules and to eliminate wild-type genomes, the primary transformants were subjected to 3 additional rounds of regeneration on selective medium. Since segregation leads to the occurrence of white, mixed and green sectors, material from white sectors was subjected to several additional rounds of regeneration on non-selective medium in order to obtain homoplastomic mutant transformants. Homoplastomic transformed lines were rooted and propagated on solid VBW-medium (Aviv and Galun, 1985) (preparation see below) under low light condition to obtain wild-type-similar *Δycf3* mutants (display light green phenotype).

RMOP (pH5.8 with KOH): NH<sub>4</sub>NO<sub>3</sub> (1650 µg/ml), KNO<sub>3</sub> (1900 µg/ml), CaCl<sub>2</sub>·2H<sub>2</sub>O 440 (µg/ml), MgSO<sub>4</sub>·7H<sub>2</sub>O (370 µg/ml), KH<sub>2</sub>PO<sub>4</sub> (170 µg/ml), EDTA-Fe(III)Na (40 µg/ml), KI (0.83 µg/ml),

H<sub>3</sub>BO<sub>3</sub> (6.2 µg/ml), MnSO<sub>4</sub>×H<sub>2</sub>O (22.3 µg/ml), ZnSO<sub>4</sub>×7H<sub>2</sub>O (8.6 µg/ml), Na<sub>2</sub>MoO<sub>4</sub>×2H<sub>2</sub>O (0.25 µg/ml), CuSO<sub>4</sub>×5H<sub>2</sub>O (0.025 µg/ml), CoCl<sub>2</sub>×6H<sub>2</sub>O (0.025 µg/ml), Inositol (100 µg/ml), Thiamine-HCl (1 µg/ml), Benzylaminopurine (1 µg/ml), Naphthalene acetic acid (0.1 µg/ml), Sucrose (30000 µg/ml), Agar, purified (8000 µg/ml).

B5 (pH5.7 with KOH): KNO<sub>3</sub> (2500 µg/ml), CaCl<sub>2</sub>×2H<sub>2</sub>O (150 µg/ml), MgSO<sub>4</sub>×7H<sub>2</sub>O (250 µg/ml), NaH<sub>2</sub>PO<sub>4</sub>×H<sub>2</sub>O (150 µg/ml), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (134 µg/ml), EDTA-Fe(III)Na (40 µg/ml), KI (0.75 µg/ml), H<sub>3</sub>BO<sub>3</sub> (3 µg/ml), MnSO<sub>4</sub>×H<sub>2</sub>O (10 µg/ml), ZnSO<sub>4</sub>×7H<sub>2</sub>O (2 µg/ml), Na<sub>2</sub>MoO<sub>4</sub>×2H<sub>2</sub>O (0.25 µg/ml), CuSO<sub>4</sub>×5H<sub>2</sub>O (0.025 µg/ml), CoCl<sub>2</sub>×6H<sub>2</sub>O (0.025 µg/ml), Inositol (100 µg/ml), Pyridoxine-HCl (1 µg/ml), Thiamine-HCl (10 µg/ml), Nicotinic acid (1 µg/ml), Sucrose (20000 µg/ml), Agar, purified (7000 µg/ml).

VBW (pH5.8 with KOH): NH<sub>4</sub>NO<sub>3</sub> (1650 µg/ml), KNO<sub>3</sub> (1900 µg/ml), CaCl<sub>2</sub>×2H<sub>2</sub>O (440 µg/ml), MgSO<sub>4</sub>×7H<sub>2</sub>O (370 µg/ml), KH<sub>2</sub>PO<sub>4</sub> (170 µg/ml), EDTA-Fe(III)Na (40 µg/ml), KI (0.83 µg/ml), H<sub>3</sub>BO<sub>3</sub> (6.2 µg/ml), MnSO<sub>4</sub>×H<sub>2</sub>O (22.3 µg/ml), ZnSO<sub>4</sub>×7H<sub>2</sub>O (8.6 µg/ml), Na<sub>2</sub>MoO<sub>4</sub>×2H<sub>2</sub>O (0.25 µg/ml), CuSO<sub>4</sub>×5H<sub>2</sub>O (0.025 µg/ml), CoCl<sub>2</sub>×6H<sub>2</sub>O (0.025 µg/ml), Inositol (100 µg/ml), Pyridoxin-HCl (0.5 µg/ml), Thiamine-HCl (1 µg/ml), Glycine (2 µg/ml), Nicotinic acid (0.5 µg/ml), Indolylacetic acid (2 µg/ml), Kinetin (0.2 µg/ml), Sucrose (30000 µg/ml), Caseinhydrolysate (500 µg/ml), Agar, purified (7000 µg/ml).

#### *Analysis by PCR and Southern blotting*

Plastid transformants were identified by PCR amplification. Total DNA isolated from the first regenerates of 40 independent lines were used as templates for separate PCR reactions. The method used was as follows: 100 mg fresh leaf tissues of tobacco were disrupted (2 x 1 min at 25 Hz) in 200 µl AP1 buffer (DNeasy plant mini kit, QIAGEN) / 1 µl reagent DX (foaming inhibition, QIAGEN) using mixer mill MM 300 (Retsch) in a 1.5 ml microcentrifuge tube with one 3mm tungsten carbide bead. DNA was then purified using the DNeasy plant mini kit. Five sets of primers (sequences are shown in table 1), namely oFCH59 and oFCH60; oFCH52 and oFCH53; oFCH52 and oFCH60; oFCH53 and oFCH59; oFCH60 and oFCH27 were employed to analyze transplastomic plants. oFCH52 and oFCH53 should result in an amplification product of 900 bp from the wild-type plastome and a product of 1700 bp from transformed plastomes, whereas oFCH59 and oFCH60 should result in an amplification product of 480 bp from the transformed plants and no product from wild-type. Likewise,

oFCH52, oFCH60 and oFCH53, oFCH59 should only amplify a product of 867 bp and 1368 bp from the transformed plants, respectively. The combination of oFCH60 and oFCH27 can determine whether the transformants carry correct insertions or not by amplifying a product of 2541 bp from correctly transformed plastomes.

Table 1

Primers	sequences	location
oFCH59	5'-TGC TGG CCG TAC ATT TGT ACG-3'	derived from the 5' portion of the <i>aadA</i> coding region
oFCH60	5'-CAC TAC ATT TCG CTC ATC GCC-3'	derived from the 3' portion of the <i>aadA</i> coding region
oFCH52	5'-CAC TAC ATT TCG CTC ATC GCC-3'	annealing with plastome nucleotides 45903-45922, located within cloned plastid DNA fragment
oFCH53	5'-GAC TAT AGT TAA TGG ATA CTC-3'	annealing with plastome nucleotides 46812-46792, located within cloned plastid DNA fragment
oFCH27	5'-TGC TCA AGA CTT TAG TGG ATC-3'	annealing with plastome nucleotides 44799-44819, located within chloroplast genome outside of cloned plastid DNA fragment

PCR results showed that 24 lines of transformants carried the *aadA* gene with correct insertion in the plastid genome but they were still heteroplasmic in the first cycle of regeneration. The data are also consistent with phenotypic appearance of the respective lines, which indicated that the pigment deficiency was correlated with deletion of *ycf3*.

Homoplasmy was verified by DNA gel blot analysis. Genomic DNAs isolated from young leaves from plants derived from the fourth cycle of regeneration grown under low light conditions were used for DNA gel blot analysis. The detailed procedure was as follows: 4 µg of total plant DNA per analyzed plant were digested with restriction enzyme *Xma* I and separated on a TBE-agarose gel (0.8%). The DNA was denatured and transferred to a positively charged nylon membrane (Hybond-N<sup>+</sup>, Amersham) as described in Ausubel *et al.* (1999). The filter was hybridized with digoxigenin-labeled probes in DIG Easy Hyb Buffer



(Roche Diagnostics GmbH, Mannheim, Germany), and hybridization signals were detected using the DIG Luminescent Detection Kit (Roche). The membrane was exposed to an X-OMAT LS film at room temperature for 80 minutes.

For preparation of a DIG-labeled probe, plasmid pIC522 (see below) was used as template to amplify a 520 bp fragment using the following pair of primers: oFCH69 (5'-CAT TGG AAC TGC TAT GTA GGC-3', corresponding to tobacco plastome sequence 47149-47169) and oFCH64 (5'-GAA TTA CCA AAC CAT TTG ACC C-3', corresponding to tobacco plastome sequence 47667-47647). The PCR DIG Probe Synthesis Kit from Roche was used. The PCR program was as follows: 2 min at 94°C, 1 cycle; 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C, 35 cycles; final extension at 72°C for 10 min. The amplified fragment was gel purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and then used for hybridization. This probe should result in a signal of 2998 bp from the transformed plastomes and a signal of 2198 bp from wild-type plastomes. The result showed that no wild-type plastid DNA could be detected in all 10 examined mutant lines.

#### *Construction of transformation vector pIC526 for reconstitution of the ycf3 gene*

Transformation vector pIC526 was designed to transform the mutant *Dycf3* line with the goal to reconstitute the *ycf3* gene, delete the *aadA* cassette and insert a GFP gene at the same time.

The region of the tobacco chloroplast genome containing the first exon and 5' regulatory element of *ycf3* (571 bp) was amplified from genomic DNA isolated from leaf tissue of tobacco by PCR. The following pair of oligonucleotide primers was used: oFCH48 5'-*Sma* I-*Dra* I-*Kpn* I-GTG TTT TTC TCC TCG TAA GAC-3' (annealing with plastome nucleotides 46070-46090) and oFCH49 5'-*Sma* I-*Bam* HI-*Bbr* PI-*Nhe* I-CCG TTA TGT ACA CAA AAT TG -3' (annealing with plastome nucleotides 46637-46618). The PCR program was as follows: 2 min at 94°C, 1 cycle; 45 sec at 94°C, 45 sec at 55°C, 2 min at 72°C, 30 cycles; final extension at 72°C for 10 min. The fragment was digested with *Sma* I and ligated into plasmid pIC517 (construction see above) digested with *Bbr* PI and *Bst* 1107I. A plasmid clone carrying the first exon and 5' regulatory element of *ycf3* in the correct orientation regenerated plasmid pIC522, which contains a cloned plastid DNA with additional 5 restriction sites.

The coding region of GFP was amplified from plasmid pKCZ-GFP (Fig. 6) by PCR using the following pair of primers: oFCH25 (5'-CTA GCT AGC TTA TTT GTA TAG TTC ATC

CAT-3' and oFCH26 (5'-TCC CCC GGG GCC GTC GTT CAA TGA GAA TGG-3'). The PCR program was as follows: 2 min at 94°C, 1 cycle; 45 sec at 94°C, 45 sec at 55°C, 2 min at 72°C, 30 cycles; final extension at 72°C for 10 min. The amplified GFP fragment was cut with *Sma* I and *Nhe* I, and then ligated into pIC522 cut with *Bbr* PI and *Nhe* I, generating pIC526 (Fig. 3). The identity of the plasmid insert was verified by sequencing (MWG, Munich).

*Plastid transformation of Dycf3 mutant lines and selection of homoplastomic lines*

The goal of the second transformation was to reconstitute the *ycf3* gene, remove the *aadA* marker and to introduce the *gfp* gene at the same time. Young leaves from sterile homoplastomic *Dycf3* mutants grown under low light conditions on solid VBW medium were bombarded with plasmid pIC526 coated gold particles using the Bio-Rad (Hercules, CA, USA) PDS-1000/He Biolistic particle delivery system (detailed procedure see above). Two days after bombardment, leaves were cut into small pieces (ca. 3x3 mm), transferred to solid sucrose-reduced-RMOP medium (containing 3g/liter sucrose) and cultivated under low light conditions for two weeks. Every three weeks leaf pieces were cut again, transferred to fresh medium and cultivated under strong light conditions until no further regenerates appeared. Transformants, which display a green phenotype and are able to grow photoautotrophically were selected and subjected to several additional rounds of regeneration on sucrose-reduced-RMOP medium to obtain homoplastomic tissue. Homoplastomic transplastomic lines were rooted and propagated on solid B5-medium under strong light condition.

*Molecular analysis of the secondary transplastomic plants*

Plastid transformants were identified by PCR amplification. Total DNA isolated from primary transformants which displayed green phenotype and were able to grow photoautotrophically was used as templates for PCR analysis using the following primer pair: oFCH76 (5'-GTA GCA ATC CAT TCT AGA AT-3', annealing with plastome nucleotides 46269-46288) and oFCH53 (5'-GAC TAT AGT TAA TGG ATA CTC-3', annealing with plastome nucleotides 46812-46792). This pair of oligonucleotide primers should result in an amplification product of 540 bp from the wild-type plastome, a product of 1400 bp from plastomes correctly transformed in the second round, and no product from unchanged first round transformants (since the site for p76 annealing was deleted).

Homoplasmy was verified by DNA gel blot analysis. Genomic DNA was isolated from

young leaves of plants derived from the fourth cycle of regeneration grown under strong light conditions and digested with *Ava* I. The probe used was the same as that for *Dycf3* mutants (detailed procedures for DNA blotting and hybridization see above). The probe generates a signal of 1212 bp for wild-type plastome, a signal of 2015 bp for plastomes correctly transformed in the second round, and a signal of 6852 bp for unchanged first round transformants.

To confirm the removal of the *aadA* marker a second hybridization of the blot (of which the former probe had been removed by a stripping procedure) was done using a 480 bp fragment of the *aadA*-gene as probe. For probe generation primers oFCH59 and oFCH60 (see above) were used in a PCR Dig labeling reaction according to the protocol of the supplier (Roche).

#### **Example 4: Construction of a selection system based on the inactivation of a photosynthesis related gene**

##### *Construction of transformation vector pIC558 for inactivation of the plastid encoded *petA* gene*

All cloning procedures were carried out using standard protocols as described in example 1 and in Ausubel et al., 1999.

Vector pIC558 comprises two flanking sequences derived from the tobacco plastome and an *aadA*-cassette (pUC16S *aadA* *Sma* *vollst*, Koop et al., 1996) in between. The homologous sequences are 5' and 3' regions of the *petA* gene, 1 kb each. The *aadA*-cassette replaces the *petA* gene (962 bp) and 300 bp of the *petA* 3' region.

Both flanking fragments were amplified by PCR using the following oligo pairs as primers: oSK13 (5'-GGAATTCCATATGGTATAAACTCATGTGTGTAAGAAA-3') and oSK14 (5'-TCCCCCGGGGTCCAATCATTGATCGCGAAA-3'), generating an *Nde* I and a *Sma* I site at the ends, and oSK15 (5'-TTCCCCGGGTTCTAAATAGAAAGA AAGTCAAATTTG-3') and oSK16 (5'-CATGCATGCGAATGAATAAGATTCTCTTAGCTC-3'), generating a *Sma* I and a *Sph* I site at the fragment ends. The PCR program used was as follows: 3 min at 94°C, 1 cycle; 45 sec at 94°C, 45 sec at 55°C, 1.5 min at 72°C, 30 cycles; final extension at 72°C for 10 min. The digested fragments (left/right flank) and the *aadA*-cassette as *Sma* I fragment were cloned in one step into the pUC19 vector which was digested with *Nde* I and *Sph* I. Construct pIC558 was analyzed by restriction experiments. The PCR amplified fragment were sequenced to prove the correct sequence of the flanking regions.

Transformation vector pIC558 is shown in figures 8 and 9.

*Construction of transformation vector pIC597, pIC599 and pIC600 for reconstitution of the petA gene*

The aim of the second transformation is to cure the *petA* inactivation and insert a new gene of interest (*uidA* or *aphA-6*, potentially *npt II*) into the plastome simultaneously. Therefore, the *petA* gene and a gene cassette (containing 5'/3' regulatory elements) were cloned in between the left/right flanking sequences. Vector pIC597 (*uidA*-cassette) comprises the same flanking sequences as vector pIC558, the *petA* gene and the *uidA* gene-cassette.

A fragment of ~2.2 kb containing 1 kb left flank, the *petA* gene sequence (962 bp) and 300 bp of the 3' region of the *petA* gene were amplified by PCR using the following oligo pair as primers: oSK13 (5'-GGAATTCCATATGGTATAAACTCATGTGTGTAAGAA-3') and oSK71 (5'-TCCCCCGGGTAGAAAACCTATTGATACGTCTTATGG-3'), generating an Nde I and a Sma I site at the fragment ends. The PCR program used was as follows: 3 min at 94°C, 1 cycle; 45 sec at 94°C, 45 sec at 55°C, 3 min at 72°C, 30 cycles; final extension at 72°C for 10 min. This fragment and the right flank were cloned together into pUC19. This vector pIC651 ('*petA*+1kb5'+1,3kb3'') comprises 1 kb left flank, the *petA* coding sequence, 300 bp of the 3' region and 1 kb right flank corresponding to *Nicotiana tabacum* plastome sequence 63.335 - 66.597.

The new gene of interest (either *uidA*, Koop et al., 1996; or *aphA-6*, vector pSK.KmR, Bateman and Purton, 2000; or *npt II*, Töpfer et al., 1987) was introduced as gene cassette (containing 5'/3' regulatory elements) between both flanking fragments. The *uidA*-cassette (as Sma I fragment) was taken from vector pIC562 ('pUC16SRBSuidA3'rbcl', Koop et al., 1996). The genes *aphA-6* and *npt II* were cloned into vector pIC562 replacing the *uidA*-gene, each. After this cloning step the *aphA-6*-cassette and an *npt II*-cassette could be isolated by Sma I digestion, respectively. These cassettes were cloned into the *petA* 3' region (insertions site 300 bp downstream to *petA*). These vectors are named '*petA*-cure-plasmids' (pIC597 with *uidA*; pIC599 with *aph6*; pIC600 with *nptII*).

The constructs were analyzed by restriction experiments and PCR amplified fragments were sequenced to prove the correct sequence of the flanking regions.

A schematic representation of the three vectors is given in figure 10. Transformation vector pIC597 is shown in figure 11.

*Primary transformation and selection of homoplastic DpetA mutants.*

Plastid transformation by particle gun with vector pIC558 and selection was carried out as described in example 3. PEG mediated plastid transformation with vector pIC558 and selection was carried out as described in example 1.

*Secondary transformation and selection of reconstituted homoplastomic DpetA mutants.*

Plastid transformation by particle gun with vector pIC558 was carried out as described in example 3. PEG mediated plastid transformation with vector pIC588 was carried out as described in example 1. Selection of transformants was done

- a) on RMOP medium with reduced sucrose content (0.3%). Transformants with a reconstitution of the *petA* knockout should be able to use photosynthetic energy for growing.
- b) on RMOP medium containing kanamycin as selection agent (gene products of *aph-6* and *nptII* detoxify kanamycin).

Transformants showed a decrease of hcf (high chlorophyll fluorescence) during repeated cycles of regeneration.

*Analysis of transformants by PCR and Southern blotting after primary transformation*

For plant DNA isolation, PCR analysis and southern blotting standard protocols were used as described in example 1. For determination of the *aadA* gene primers oFCH59-aadA480-li and oFCH60-aadA480-re (5'-CAC TAC ATT TCG CTC ATC GCC-3') were used. To determine whether the transformants carry correct insertions, primers oFCH60-aadA480-re and oSK116-petA-re (5'-AAAATAGATTCATTAGTCCGATACC-3') are used. Primer oSK116-petA-re is located upstream (outside) of the 5' flanking fragment. The PCR program used was as follows: 3 min at 94°C, 1 cycle; 45 sec at 94°C, 45 sec at 55°C, 2 min at 72°C, 30 cycles; final extension at 72°C for 10 min.

First PCR results showed that 12 lines of transformants are carrying the *aadA* gene with correct insertion in the plastid genome. Further testing and southern analysis to show whether the lines are homoplastomic or heteroplastomic are carried out as described in example 1.

*Analysis of transformants by PCR and Southern blotting after secondary transformation*

For plant DNA isolation and PCR analysis standard protocols were used as described in example 1. For determination of the *uidA* gene primers oSM61-GUS-N (5'-TCACACCGATA CCATCAGCG-3') and oSM62-GUS-C (5'-ATTGTTTGCCTCCCTGCTGC-3') were used; to determine

whether the transformants carry correct insertions primers oSM61-GUS-N (5'-TCACACCGATACCATCAGCG-3') and oSK138-petA-3'-re (5'-AATCGTAACCACTC TCTACTGG-3') are used. The PCR program used was as follows: 3 min at 94°C, 1 cycle; 45 sec at 94°C, 45 sec at 55°C, 2 min at 72°C, 30 cycles; final extension at 72°C for 10 min.

For detection of the *aph-6* gene and the *nptII* gene specific primers were used. To determine whether the transformants carry correct insertions one gene specific primer and primer oSK138-petA-3'-re are used.

Southern blotting analysis are carried out as described in example 1 and in standard protocols.

#### **Example 5: Selection for paraquat tolerance**

##### *Plant transformation and selection for paraquat resistance*

4 leaf pieces were transformed each with 1 µg pIC558 (figure 8) as described in example 4. After bombardment the leaf pieces were incubated for 2 days at 25°C on RMOP-medium.

Two days after bombardment leaves were cut into small pieces (ca. 3×3 mm), transferred to fresh RMOP-medium and incubated for 10 days in the dark at 25 °C. Then leaf pieces were cut again, transferred to fresh medium containing 5 mg/l paraquat and incubated for 10 days in the light at 25 °C. The leaf pieces were cut again, transferred to fresh medium containing 8 mg/l paraquat and incubated for 12 days in the light at 25 °C. Green regenerates from the bottom side were retrieved and transferred to individual plates containing RMOP with 8 mg/l paraquat. The lines were subjected to repeated cycles of shoot generation by cutting small leaf pieces, which form new regenerates on RMOP-medium with 8 mg/l paraquat.

##### *Molecular analysis of potential plastid transformants by Southern analysis*

3 mg of total plant DNA per analysed plant are digested with the appropriate restriction enzyme and separated on a TBE-agarose gel (1%). The DNA is denatured and transferred to a positively charged nylon membrane (Hybond-N+, Amersham) as described in Ausubel et al., 1999: Short protocols in molecular biology, Wiley, 4<sup>th</sup> edition, Unit 2.9A. The filter is hybridised with digoxigenin-labelled probes in DIG Easy Hyb Buffer (Roche Diagnostics GmbH, Mannheim, Germany), and hybridisation signals are detected using the DIG Luminescent Detection Kit (Roche). The membrane is exposed to a X-OMAT LS film at room temperature.

A fragment suitable for discrimination between wild type and transformed plastome is gel purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), labelled with digoxigenin using the Roche DIG DNA Labelling Kit and used for hybridisation.

#### **Example 6 : Reconstitution of *ycf3* using kanamycin selection**

##### *Construction of transformation vector pIC577 for targeted inactivation of the *ycf3* gene*

A transformation vector was constructed designed to inactivate the *ycf3* gene by replacing the first exon and the splicing site of *ycf3* (corresponding to plastome nucleotides 46042-46206) with the *aadA* coding region. This vector does not contain any 3' regulatory elements (neither for the *aadA* marker gene, nor for the endogenous *ycf3* or tRNA gene). In addition, no promoter elements were introduced, and the *aadA* gene is expected to be transcribed and translated by the endogenous *ycf3* upstream regulatory element.

This vector contains the *aadA* coding region, flanked by 5'- and 3'-homologous sequences which were amplified from the tobacco chloroplast genome by PCR using the following two pairs of primers: oFCH76 (5'-*Nco* I-GTA GCA ATC CAT TCT AGA AT-3', annealing with plastome nucleotides 46269-46288) and oFCH77 (5'-*Sma* I-CGG AAA GAG AGG GAT TCT AAC-3', annealing with plastome nucleotides 47205-46185); oFCH78 (5'-*Sph* I-GAA GTT TCT TTC TTT GCT ACA-3', annealing with plastome nucleotides 45033-45053) and oFCH79 (5'-*Pst* I-TAC GCT TTT T GA AGG TGA AGT-3', annealing with plastome nucleotides 46041-46021).

The PCR amplification using *Pfu* polymerase (Promega) was performed as follows: 2 min at 94°C, 1 cycle; 45 sec at 94°C, 45 sec at 55°C, 2 min at 72°C, 30 cycles; final extension at 72°C for 10 min. The amplified 5'-homologous fragment (corresponding to plastome nucleotides 46269-47205), containing 936 nucleotides upstream of the *ycf3* start codon, was digested with *Sma* I and *Nco* I and then ligated into pUC16*SaadA* plasmid (Koop *et al.*, 1996) which was digested with *Eco* RI, followed by a fill-in reaction using Klenow polymerase (Promega) and then digested with *Nco* I, generating pIC565. The amplified 3'-homologous fragment (corresponding to plastome nucleotides 45033-46041), containing 1000 nucleotides of the *ycf3* gene, was digested with *Pst* I and *Sph* I, and then ligated into pIC565 cut with *Pst* I and *Sph* I, yielding the final transformation vector pIC577 (Fig. 12 and 13). The identity of the plasmid insert was verified by sequencing (MWG, Munich).

*Primary transformation and selection of homoplastomic  $\Delta ycf3$  mutants*

Young leaves from sterile tobacco plants (cultivation see example 3) were bombarded with plasmid pIC577-coated gold particles using the Bio-Rad (Hercules, CA, USA) PDS-1000/He Biolistic particle delivery system (detailed procedure see example 3). Two days after bombardment, leaves were cut into small pieces (ca. 3×3 mm) and transferred to solid RMOP-medium containing 500 µg/ml spectinomycin. Leaf pieces were cut again and transferred to fresh medium after 2 weeks, then every 3 weeks until no further regenerants appeared. Primary  $\Delta ycf3$  transformants displayed spectinomycin-resistance and a green phenotype in the light while still being heteroplastomic. In order to amplify transformed plastid DNA molecules and to eliminate wild-type genomes, the primary transformants were subjected to 3 additional rounds of regeneration on selective medium. Since segregation leads to the occurrence of white and green sectors, material from white sectors was subjected to several additional rounds of regeneration on non-selective medium in order to obtain homoplastomic mutant transformants. Homoplastomic transformed lines were rooted and propagated on solid VBW-medium (Aviv and Galun, 1985; see example 3).

*Analysis by PCR and Southern blotting*

Plastid transformants were identified by PCR amplification. The total DNA isolated from the first regenerates of 24 independent lines were used as a template for PCR. Two sets of primers (the sequences see example 3): oFCH59 and oFCH60; oFCH52 and oFCH53 were employed to analyze transplastomic plants. oFCH52 and oFCH53 should result in an amplification product of 900 bp from the wild-type plastome and a product of 1476 bp from transformed plastomes, whereas oFCH59 and oFCH60 should result in an amplification product of 480 bp from the transformed plants and no product from wild-type. The results show that 14 lines of transformants carry correct *aadA* insertions in the plastid genome. The data are also consistent with phenotypic appearance of the respective lines, which indicated that the pigment deficiency was correlated with deletion of *ycf3*.

Homoplasmy was verified by DNA gel blot analysis. Genomic DNAs isolated from young leaves of  $\Delta ycf3$  mutants (4<sup>th</sup> regenerates) grown under low light conditions were used for DNA gel blot analysis. Detailed procedure was as follows: 4 µg of total plant DNA per analyzed plant was digested with restriction enzyme *Xma* I and separated on a TAE-agarosegel (0.8%). The DNA was denatured and transferred to a positively charged nylon membrane



(Hybond-N<sup>+</sup>, Amersham) as described in Ausubel *et al.* (1999). The filter was hybridized with digoxigenin-labeled probes in DIG Easy Hyb Buffer (Roche Diagnostics GmbH, Mannheim, Germany), and hybridization signals were detected using the DIG Luminescent Detection Kit (Roche). The membrane was exposed to a X-OMAT LS film at room temperature for 2 hours.

For preparation of a DIG-labeled probe, tobacco genomic DNA was used as template to amplify a 520 bp fragment using the following pair of primers: oFCH69 (5'-CAT TGG AAC TGC TAT GTA GGC-3', corresponding to tobacco plastome sequence 47149-47169) and oFCH64 (5'-GAA TTA CCA AAC CAT TTG ACC C-3', corresponding to tobacco plastome sequence 47667-47647). The PCR DIG Probe Synthesis Kit from Roche was used. The PCR program was as follows: 2 min at 94°C, 1 cycle; 30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C, 30 cycles; final extension at 72°C for 10 min. The amplified fragment was gel purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and then used for hybridization. This probe should result in a signal of 2780 bp from the transformed plastomes and a signal of 2198 bp from wild-type plastomes. The result showed that no wild-type plastid DNA could be detected in all 6 examined mutant lines.

#### *Construction of the transformation vector pIC637 for reconstitution of the ycf3 gene*

Transformation vector pIC637 was designed to transform the mutant  $\Delta ycf3$  line with the goal to reconstitute the *ycf3* gene, delete the *aadA* gene and insert the *aphA-6* gene that confers resistance to kanamycin at the same time.

The *aphA-6* gene is introduced into the upstream position of *ycf3* without disruption of either *ycf3* expression or the function of the endogenous *ycf3* upstream regulatory element. A short RBS (ribosomal bonding site) sequence serves as the signal to translate the reconstituted *ycf3* gene as a newly formed artificial operon. The *aphA-6* gene and *ycf3* are transcribed in the same direction under control of *ycf3* 5'-regulatory element.

The region of the tobacco chloroplast genome (corresponding to plastome nucleotides 45033-46266) containing the N-terminal of *ycf3* (which is deleted in the first round transformation) was amplified from genomic DNA isolated from leaf tissue of tobacco by PCR. The following pair of oligonucleotide primers were used: oFCH139 (5'-*Pst* I-ATC ACT AGT TGT AGG GAG GGA TCC (ribosome binding site)-ATG CCT AGA TCA CGG ATA AA -3', annealing with plastome nucleotides 46266-46247) and oFCH78 (5'-*Sph* I-GAA GTT TCT TTC TTT GCT ACA-3', annealing with plastome nucleotides 45033-45053). The PCR amplification using *Taq* polymerase (Promega) was performed as follows: 2 min at 94°C, 1 cycle; 45 sec at

94°C, 45 sec at 55°C, 2 min at 72°C, 30 cycles; final extension at 72°C for 10 min. The fragment was digested with *Pst* I and *Sph* I, and then ligated into pIC577 cut with *Pst* I and *Sph* I, generating pIC636.

The coding region of the *aphA-6* gene was cut from the plasmid pSK.KmR (obtained from Dr. Saul Purton, Department of Biology University college London, UK) using *Nco* I and *Pst* I and then ligated into pIC636 cut with *Nco* I and *Pst* I, yielding the final transformation vector pIC637 (Fig. 14 and 15). The identity of the plasmid insert was verified by sequencing (MWG, Munich).

#### *Plastid transformation of $\Delta ycf3$ mutant lines and selection of homoplastomic lines*

The goal of the second transformation was to reconstitute the *ycf3* gene, remove the *aadA* marker and introduce the *aphA-6* gene that confers resistance to kanamycin at the same time. Embedded protoplasts isolated from sterile homoplastomic  $\Delta ycf3$  mutants grown under low light conditions on solid VBW-medium were bombarded with plasmid pIC637-coated gold particles using the Bio-Rad (Hercules, CA, USA) PDS-1000/He Biolistic particle delivery system (detailed procedure see example 3). Two days after bombardment, grids were transferred to solid RMOP medium, containing 25 µg/ml kanamycin and cultivated under low light conditions for two weeks. Afterwards, every two weeks grids were transferred to fresh medium and cultivated under strong light conditions until no further regenerates appeared. The transformants which display kanamycin resistance and a green phenotype were selected and subjected to B5 medium under strong light condition to amplify *ycf3*-reconstituted plastomes (*ycf3*-deficient plastomes can not be amplified when growing on B5 medium and strong light conditions).

#### *Molecular analysis of the secondary transplastomic plants*

Plastid transformants were identified by PCR amplification. The total DNA isolated from primary transformants which displayed green phenotype and were able to grow photoautotrophically were used as a template for PCR analysis using the following two pairs of primers: oFCH168 (5'-TCA GTC GCC ATC GGA TGT TT-3', derived from the 5' portion of the *aphA-6* coding region) and oFCH169 (5'-ACC AAT CTT TCT TCA ACA CG -3', derived from the 3' portion of the *aphA-6* coding region); oFCH27 (5'-TGC TCA AGA CTT TAG TGG ATC-3', annealing with plastome nucleotides 44799-44819) and oFCH168. oFCH168 and

oFCH169 should result in an amplification product of 500 bp from the reconstituted plants and no product from unchanged first round transformants. The combination of oFCH27 and oFCH168 can determine whether the second round transformants carry correct *aphA-6* insertions or not by amplifying a product of about 2300 bp from correctly transformed plastomes. In total 5 unique *ycf3*-reconstituted tobacco-plastid transformants were obtained from 3 grid bombardments.

Homoplasmy was verified by DNA gel blot analysis. Genomic DNA was isolated from young leaves of *ycf3*-reconstituted plants grown on B5 medium under strong light conditions and digested with *Hinc* II. The probe used was the same as that for  $\Delta ycf3$  mutants (detailed procedures for DNA blotting and hybridization see above). The probe generates a signal of 3257 bp for wild-type plastome, a signal of 2046 bp for plastomes correctly transformed in the second round, and a signal of 3857 bp for unchanged first round transformants.

To confirm the removal of the *aadA* marker a second hybridization of the blot (of which the former probe had been removed by a stripping procedure) was done using a 480 bp fragment of the *aadA*-gene as probe. For probe generation primers oFCH59 and oFCH60 (see above) were used in a PCR DIG labeling reaction according to the protocol of the supplier (Roche).

## REFERENCES

- Allison LA, Simon LD, Maliga P 1996, EMBO J 15: 2802-2809.
- Ausubel (ed.), Current Protocols in Molecular Biology, John Wiley and Sons, Inc. (1999).
- Ausubel et al., 1999: Short protocols in molecular biology, Wiley, 4<sup>th</sup> edition.
- Aviv, D and Galun, E 1985, J. Hered. 76: 135-136.
- Bock R, Kössel H, Maliga P 1994, EMBO J 13:4623-8.
- Boynton J.E., Gillham N.W., Harris E.H., Hosler J.P., Johnson A.M., Jones A.R., Randolph-Anderson B.L., Robertson D., Klein T.M., Shark K.B., et al. (1988), Science, 240, 1534-1538.
- Carrer H., Hockenberry T.N., Svab Z., Maliga P. 1993, Mol Gen Genet 241: 49-56.
- Daniell H., Datta R., Varma S., Gray S., Lee S.B., 1998, Nat. Biotech., 16, 345-348.
- De Santis-Maclossek G, Kofer W, Bock A, Schoch S, Maier RM, Wanner G, Rüdiger W, Koop HU, Hermann RG 1999, Plant J. 18:477-89.
- Dovzhenko A, Bergen U, Koop HU 1998, Protoplasma 204, 114-18.

- Eibl C, Zou Z, Beck a, Kim M, Mullet J, Koop HU 1999, *Plant J.*, 19, 333-345.
- Fischer N, Stampacchia O, Redding K, and Rochaix JD, 1996, *Mol Gen Genet* 251, 373-380.
- Galvin S. B., 1998, *Curr. Opin. Biotechnol.*, 9, 227-232.
- Goldschmidt-Clermont (1991) *Nucl Acids Res* 19, 4083-4089.
- Gray M. W., Origin and Evolution of Plastid Genomes and Genes, in: Bogorad L. and Vasil I. K. (eds.), *Cell Culture and Somatic Cell Genetics of Plants, Volume 7A*, Academic Press, San Diego, 1991.
- Heifetz, 2000, *Biochimie*, 82, 655-666.
- Hock and Elstner, 1995: *Schadwirkung auf Pflanzen; Spektrum*, 3. Auflage; pages 155-186
- Iamtham, S, Day, A., 2000, *Nature Biotechnology* 18, 1172-1176.
- Kavanagh T.A., Thanh N.D., Lao N.T., McGrath N., Peter S.O., Horvath E.M., Dix P.J., Medgyesy P., 1999, *Genetics*, 152, 1111-1122.
- Khan MS, Maliga P, *Nat Biotechnol* 1999, 17:910-5.
- Koop HU, Steinmüller K, Wagner H, Rössler C, Eibl C, Sacher L 1996, *Planta*, 199: 193-201.
- Krause K, Maier RM, Kofer W, Krupinska K, Herrmann RG 2000, *Mol. Gen. Genet.* 263: 1022-1030.
- Maniatis, E. F. Fritsch and J. Sambrook, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor laboratory, Cold Spring Harbor, NY (1989).
- Marechal-Drouard L., Kuntz M., Weil J. H., tRNAs and tRNA Genes of Plastids, in: Bogorad L. and Vasil I. K. (eds.), *Cell Culture and Somatic Cell Genetics of Plants, Volume 7A*, Academic Press, San Diego, 1991.
- Monde RA, Greene JC, Stern DB 2000 *Mol Gen Genet* 263(4):610-8.
- Monde RA, Zito F, Olive J, Wollman FA, Stern DB 2000 *Plant J* :61-72.
- Palmer J. D., Plastid Chromosomes: Structure and Evolution, in: Bogorad L. and Vasil I. K.(eds.), *Cell Culture and Somatic Cell Genetics of Plants, Volume 7A*, Academic Press, San Diego, 1991.
- Ruf S, Kössel H, Bock R1997, *J. Cell Biol.* 139: 95-102.
- Silhavy, M. L. Berman, and L. W. Enquist, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984).
- Svab Z, Hajdukiewicz, P, Maliga P 1990, *Proc Natl Acad Sci USA* 87:8526-8530.
- Svab Z. & Maliga P., 1993, *Proc. Natl. Acad. Sci. USA* 90, 913-917.
- The Arabidopsis Genome Initiative, 2000, *Nature* 408: 796-815.

### Claims

1. A process for producing multicellular plants, plant organs or plant tissues transformed on their plastome by the following steps:
  - (a) altering or disrupting the function of a gene in a plastid genome for producing a selectable or recognizable phenotype;
  - (b) separating or selecting plants or cells having plastids expressing said phenotype;
  - (c) transforming said plastid genome of said separated or selected plant, plant organ or plant tissue with at least one transformation vector having a restoring sequence capable of restoring said function; and
  - (d) separating or selecting said transformed plant, plant organ or plant tissue having plastids expressing said restored function.
2. Process according to claim 1, wherein the transformation of step (c) restores said function in conjunction with introducing at least one additional function.
3. Process according to one of claims 1 or 2, wherein the transformation of step (c) restores said function in conjunction with causing a desired additional genetic modification of the plastid genome.
4. Process according to one of claims 1 to 3, wherein the transformation of step (c) additionally eliminates a preexisting function.
5. Process according to one of claims 1 to 4, wherein said alteration or disruption of step (a) is obtained by spontaneous or induced mutation.
6. Process according to one of claims 1 to 5, wherein said alteration or disruption of step (a) is obtained by genetic transformation.
7. Process according to claim 6, wherein said genetic transformation results simultaneously in the introduction of at least one additional sequence for at least one additional function.

8. Process according to claim 7, wherein said additional function is an inhibitor resistance function and step (b) is carried out in the presence of the corresponding inhibitor.
9. Process according to claim 8, wherein in step (b) the inhibitor is only added initially.
10. Process according to one of claims 1 to 9, wherein steps (a) and (b) alter or disrupt a trophic type and steps (c) and (d) restore the trophic type.
11. Process according to claim 10, wherein the restored trophic type is phototrophy.
12. Process according to one of claims 1 to 11, wherein steps (c) and (d) revert an inhibitor resistance or insensitivity introduced in steps (a) and (b).
13. Process according to one of claims 1 to 12, wherein the vector(s) used in step (a) and/or (c) comprise(s) a sequence having a homology to a host plastid sequence sufficient for homologous recombination.
14. Process according to one of claims 1 to 13, wherein the plant, plant organs or plant tissue is/are grown or cultured heterotrophically in step (b).
15. Process according to one of claims 1 to 14, wherein said alteration or disruption of step (a) produces a pigment deficient phenotype.
16. Process according to claim 15, wherein said pigment deficient phenotype is chlorophyll deficiency.
17. Process according to one of claims 1 to 16, wherein the gene altered or disrupted in step (a) is a plastome encoded plastid gene essential for transcription or translation, notably an RNA polymerase.
18. Process according to one of claims 1 to 17, wherein the gene altered or disrupted in step (a) is a plastid *rpoA* or *rpoB* gene.

19. Process according to one of claims 1 to 14, wherein the phenotype produced in step (a) can alternate between two or several appearances dependent on external growth conditions.
20. The process according to claim 19, wherein the gene altered or disrupted in step (a) is a plastid *ycf3* gene, producing a yellow-white phenotype under standard light conditions and a light green phenotype under low light conditions.
21. Process according to one of claims 1 to 15, wherein the gene altered or disrupted in step (a) is a plastid gene which produces a high chlorophyll fluorescence phenotype upon alteration or disruption, preferably *petA*.
22. Process according to claim 21, wherein an inhibitor that requires active photosynthesis for efficacy is used in step (b).
23. Process according to claim 22, wherein said inhibitor is paraquat, morphamquat, diquat, difenzoquat and/or cyperquat.
24. Process according to one of claims 1 to 23, wherein a photomixotrophic conditions are used in step (d).
25. Process according to one of claims 1 to 24, wherein a sequence introduced in step (a) and a sequence introduced in step (c) together result in an additional function.
26. Process according to one of claims 1 to 25, wherein step (d) is assisted by an inhibitor resistance.
27. A vector for transforming a plastid genome of a multicellular plant or cells thereof comprising a restoring sequence capable of restoring a disrupted function encoded by said plastid genome and at least one sequence being homologous to a sequence portion of said plastid genome to be transformed such that restoration of said disrupted function results from homologous recombination.

28. The vector of claim 27 having at least one additional sequence for introducing at least one additional function.
29. The vector of one of claims 27 or 28 further comprising a sequence causing a desired additional genetic modification of said plastid genome.
30. The vector of one of claims 27 to 29 having said homologous sequence(s) positioned such that a predetermined sequence portion of said plastid genome is eliminatable.
31. Multicellular plant, plant tissue or seeds thereof obtainable by the process of one of claims 1 to 26.



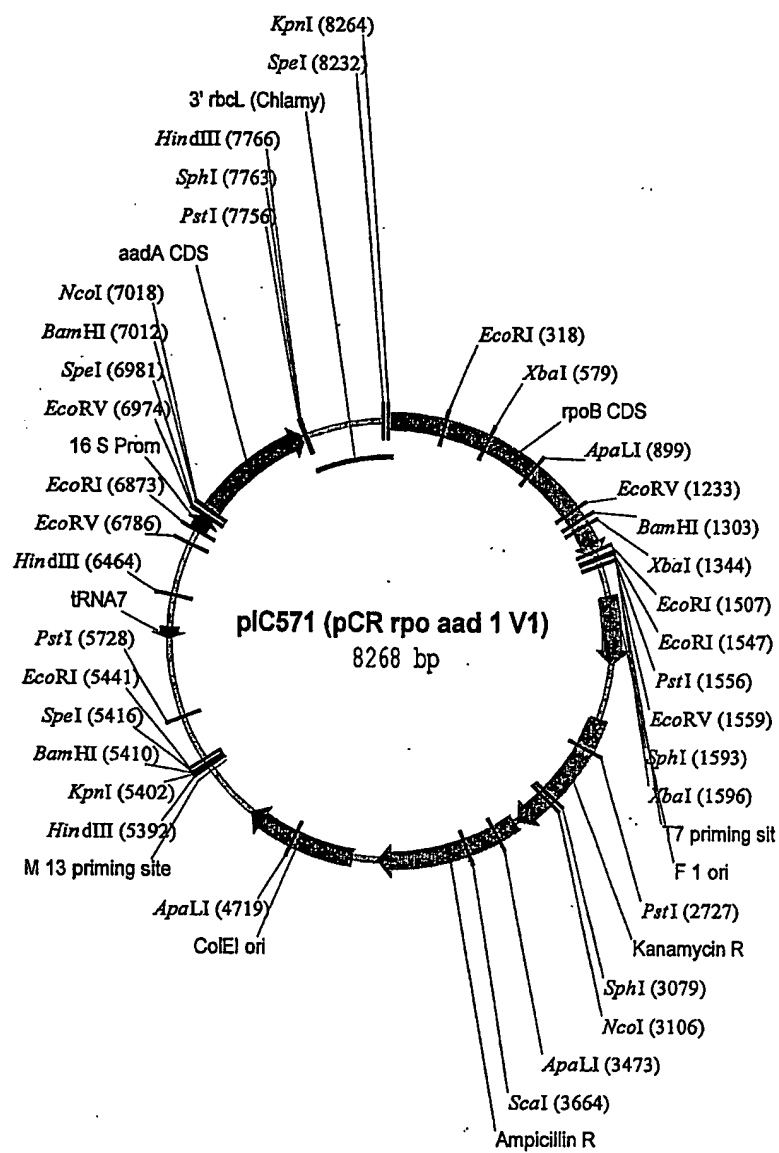


Fig. 1

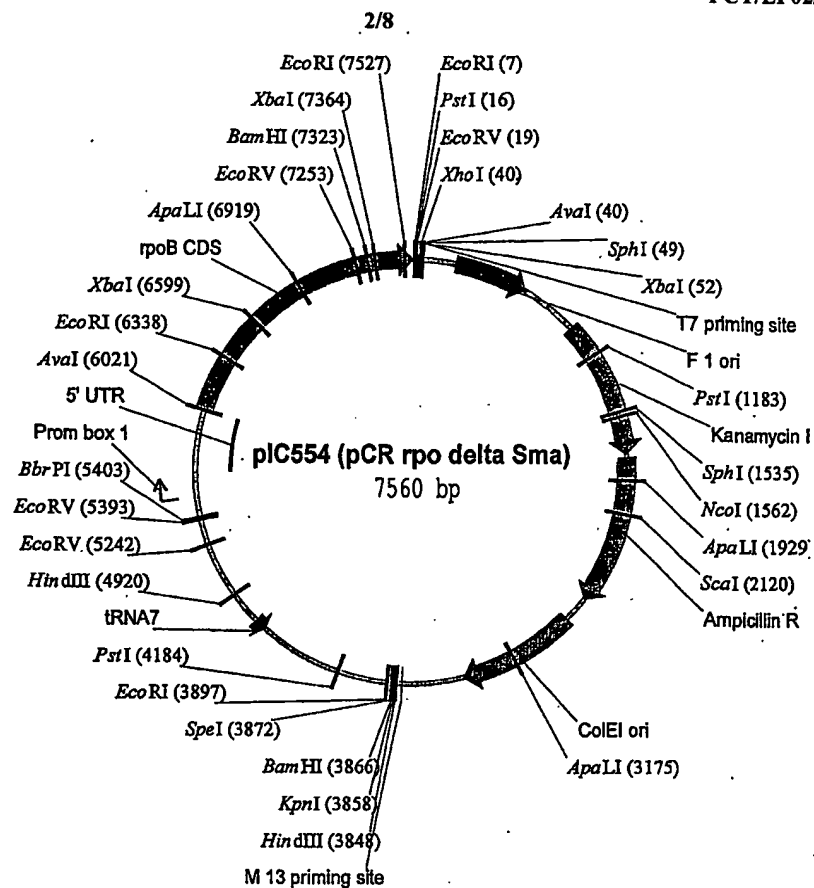


Fig. 2

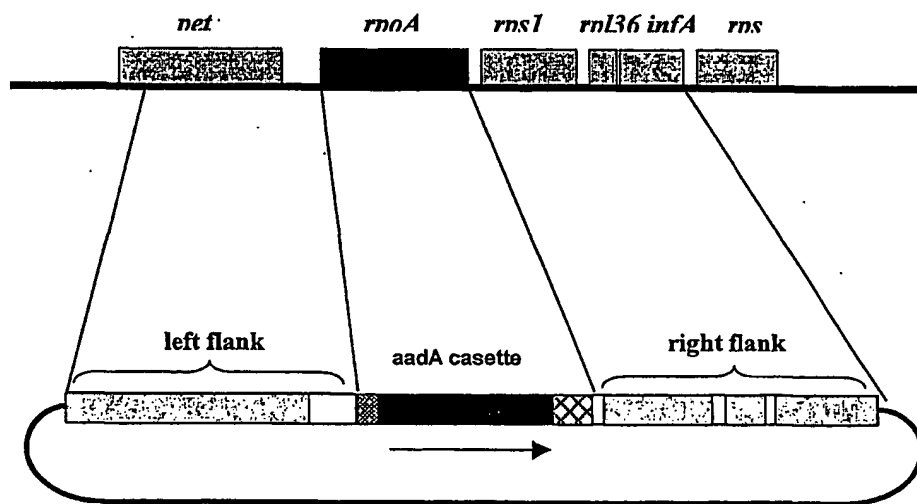


Fig. 3

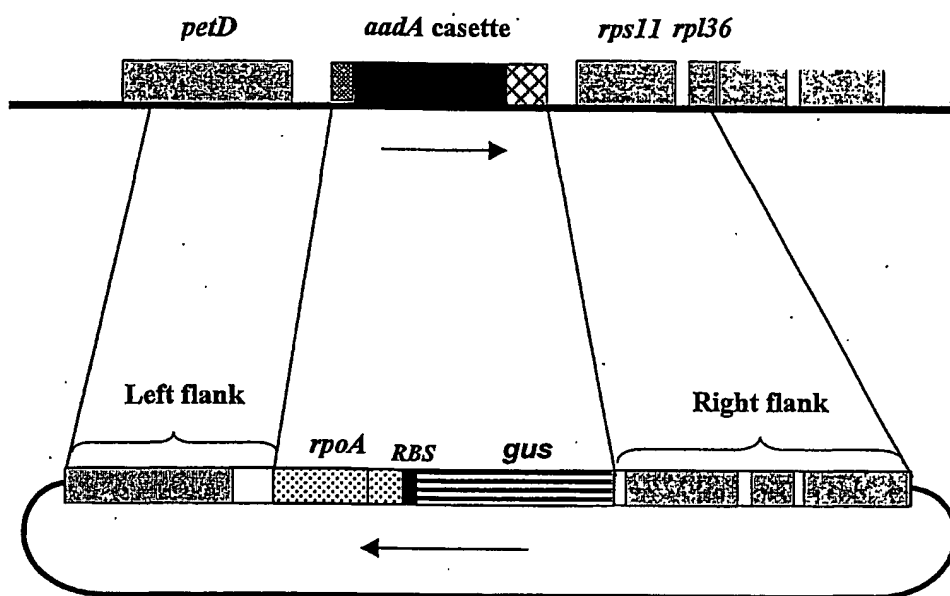


Fig. 4

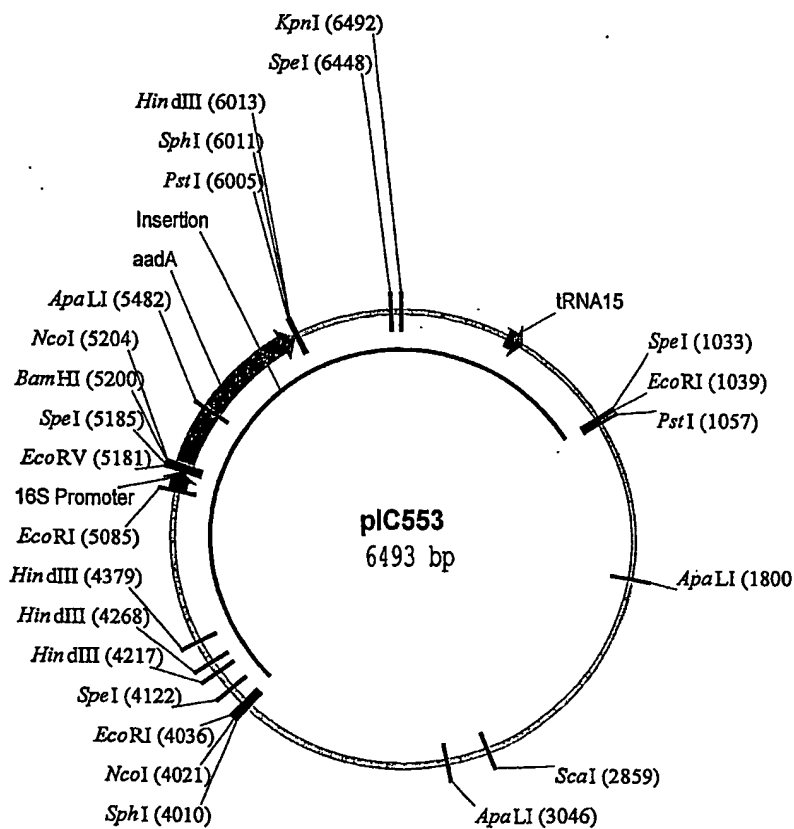


Fig. 5

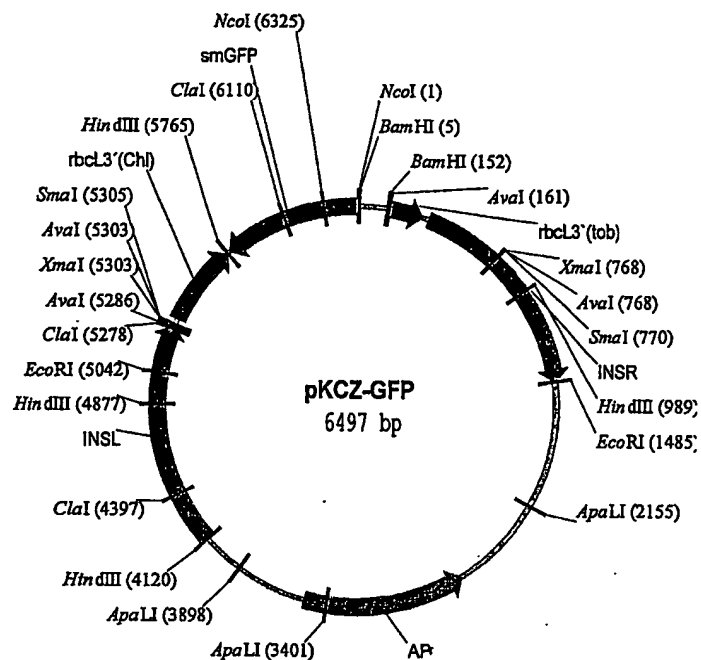


Fig. 6

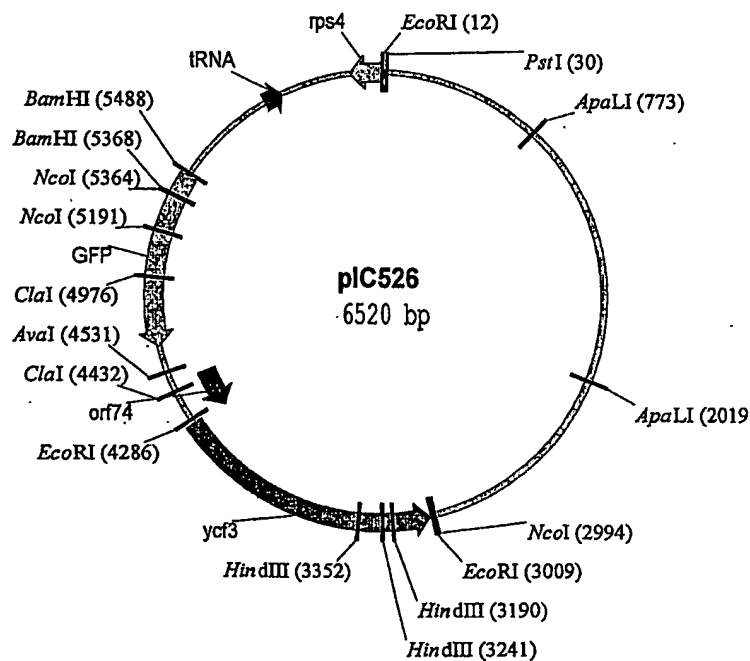


Fig. 7

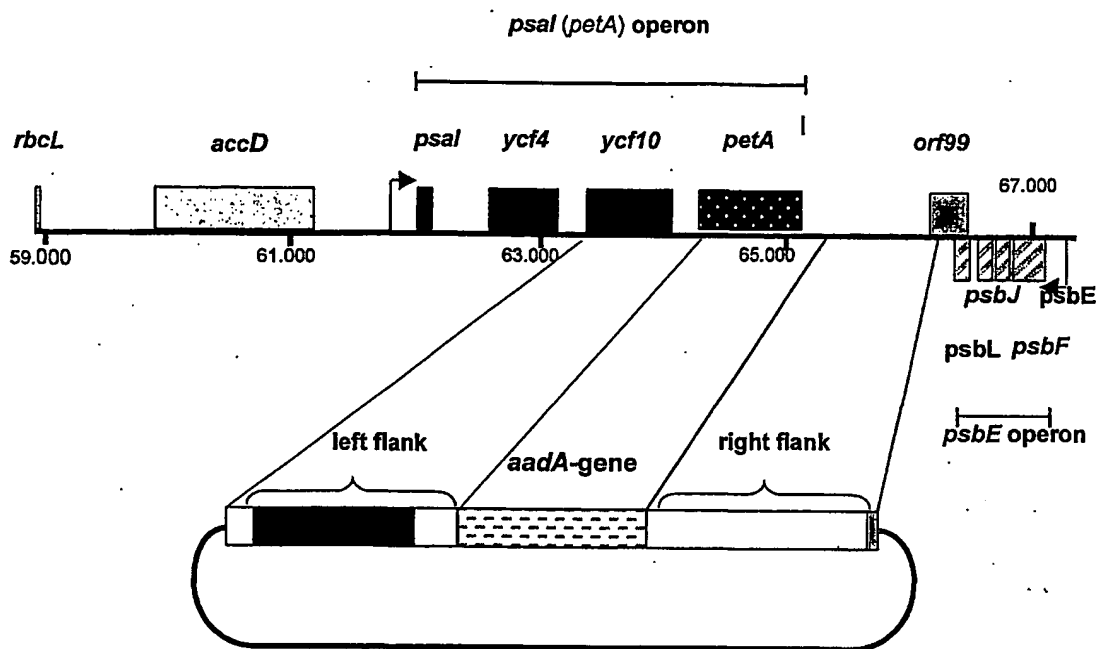


Fig. 8

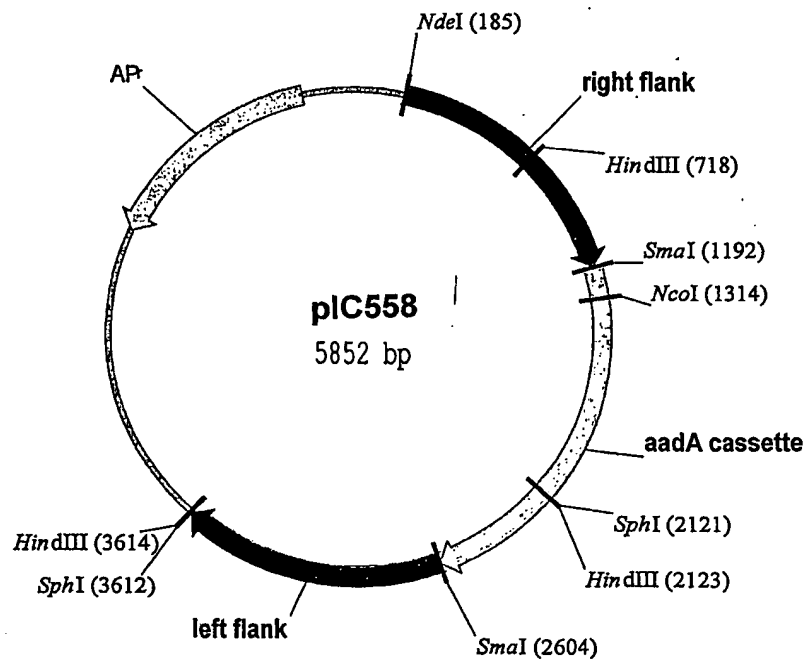


Fig. 9

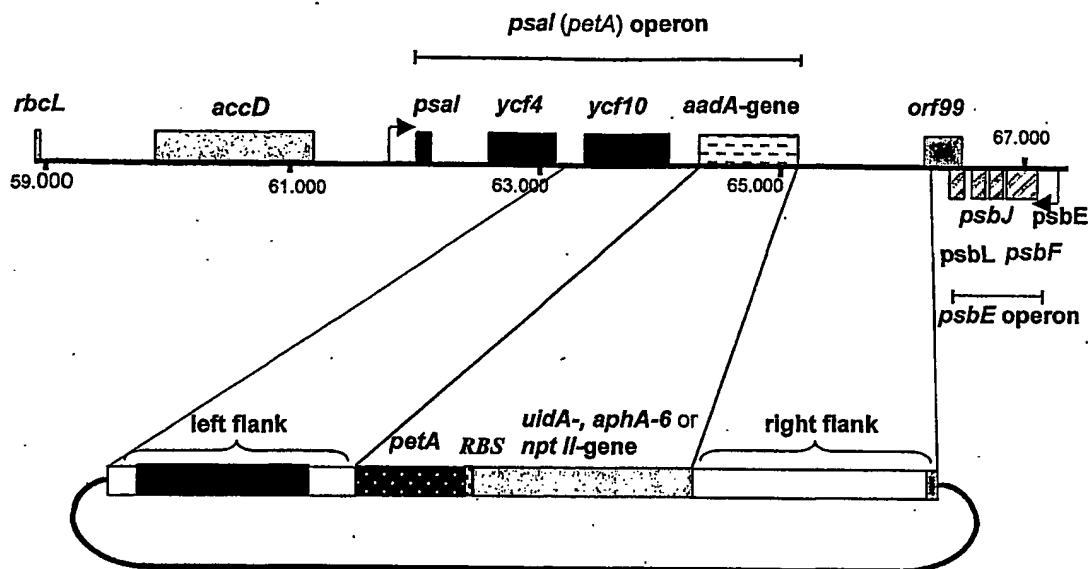


Fig. 10

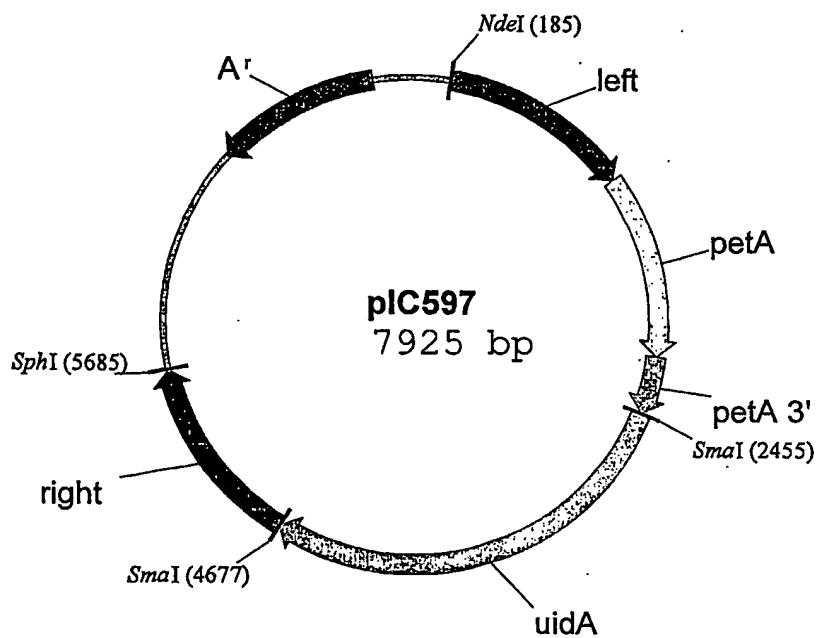


Fig. 11

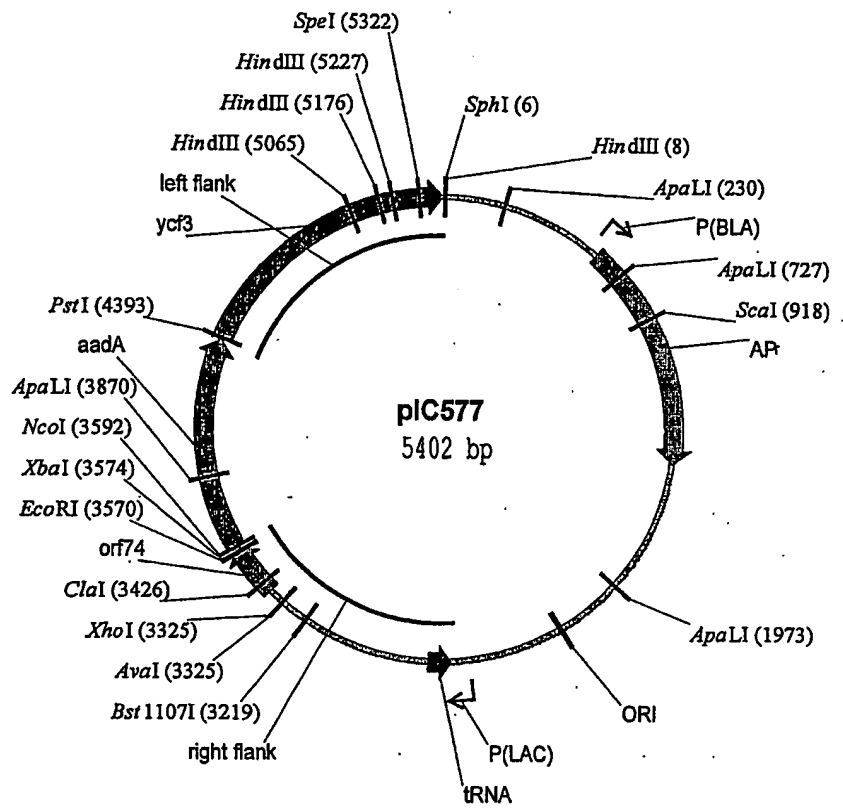


Fig. 12

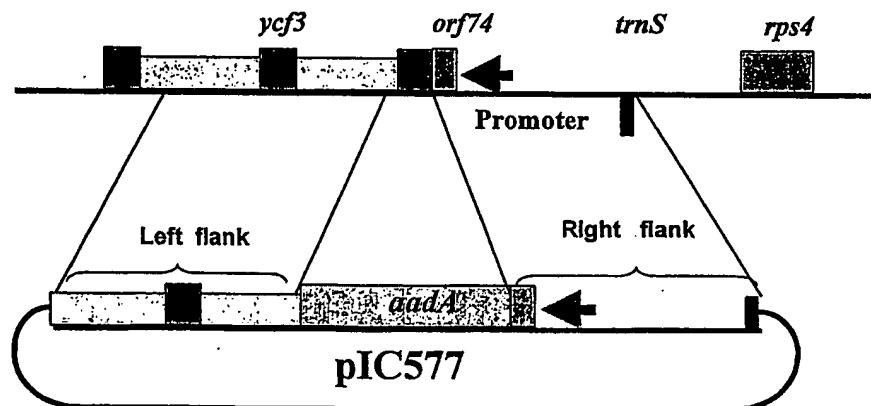


Fig. 13

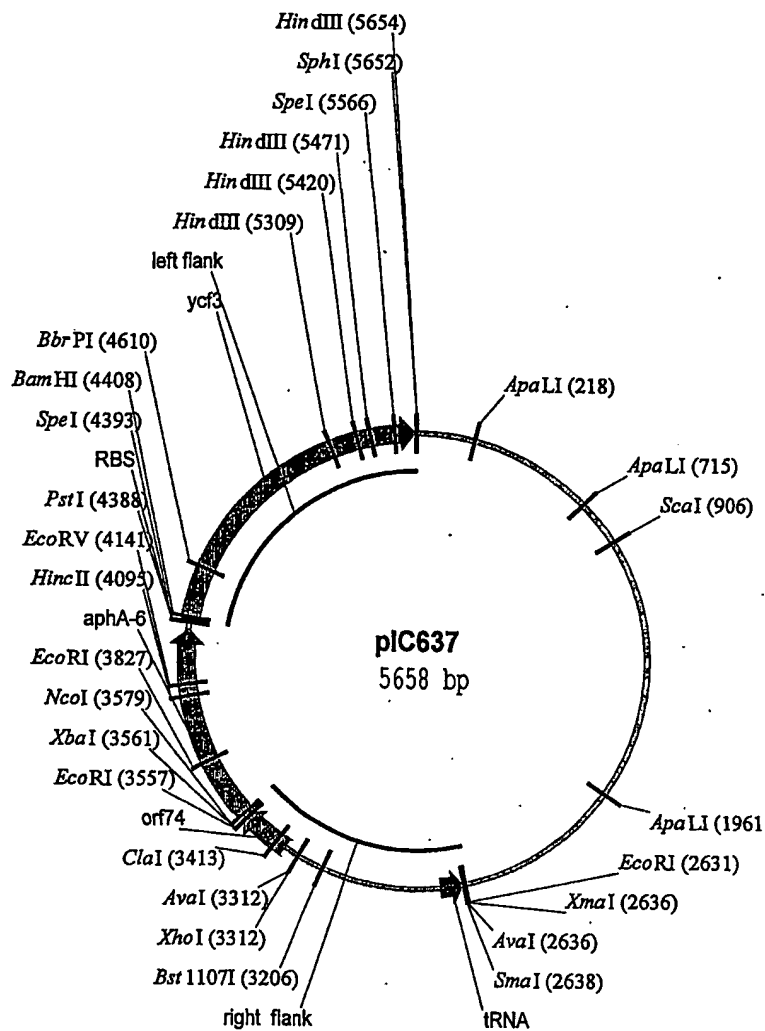


Fig. 14

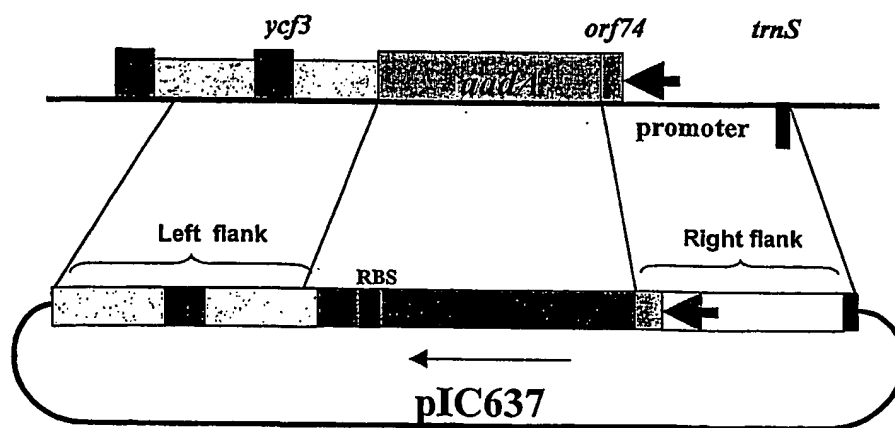


Fig. 15



(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
25 July 2002 (25.07.2002)

PCT

(10) International Publication Number  
WO 02/057466 A3

(51) International Patent Classification<sup>7</sup>: C12N 15/82,  
A01H 5/00

(21) International Application Number: PCT/EP02/00481

(22) International Filing Date: 18 January 2002 (18.01.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
101 02 389.8 19 January 2001 (19.01.2001) DE

(71) Applicant (for all designated States except US): ICON  
GENETICS AG [DE/DE]; Maximilianstrasse 38/40,  
80539 München (DE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): EIBL, Christian  
[DE/DE]; Sommerhaus, 85241 Herbertshausen (DE).  
HUANG, Fong-Chin [—/DE]; Frankfurter Ring 8, 80807  
München (DE). KLAUS, Sebastian [DE/DE]; Gräfr. 34,  
81241 München (DE). MÜHLBAUER, Stefan [DE/DE];  
Ganzenmüllerstr. 8, 85354 Freising (DE). HERZ, Stefan  
[DE/DE]; Clemensstrasse 12, 80803 München (DE).  
KOOP, Hans-Ulrich [DE/DE]; Gerastrasse 11a, 80993  
München (DE).

(74) Agent: HARTZ, Nikolai, F.; Wächterhäuser & Hartz, Tal  
29, 80331 München (DE).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG,  
SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,  
VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR,  
GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent  
(BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,  
NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments

(88) Date of publication of the international search report:  
27 February 2003

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

WO 02/057466 A3

(54) Title: PROCESSES AND VECTORS FOR PLASTID TRANSFORMATION OF HIGHER PLANTS

(57) Abstract: A process for producing multicellular plants, plant organs or plant tissues transformed on their plastome by the following steps is provided: (a) altering or disrupting the function of a gene in a plastid genome for producing a selectable or recog-  
nizable phenotype; (b) separating or selecting plants or cells having plastids expressing said phenotype; (c) transforming said plastid  
genome of said separated or selected plant, plant organ or plant tissue with at least one transformation vector having a restoring  
sequence capable of restoring said function; and (d) separating or selecting said transformed plant, plant organ or plant tissue having  
plastids expressing said restored function.

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/00481

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12N15/82 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, MEDLINE, EMBASE, SCISEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HEIFETZ P B: "Genetic engineering of the chloroplast" BIOCHIMIE, MASSON, PARIS, FR, vol. 82, 2000, pages 655-666, XP002190805 ISSN: 0300-9084 cited in the application p. 657-661	1-31
Y	BOYNTON J E ET AL: "CHLOROPLAST TRANSFORMATION IN CHLAMYDOMONAS WITH HIGH VELOCITY MICROPROJECTILES" SCIENCE (WASHINGTON D C), vol. 240, no. 4858, 1988, pages 1534-1538, XP001120602 ISSN: 0036-8075 abstract, p. 1535-1537, fig. 1,2 --- -/--	1-18, 24-31

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

Date of the actual completion of the international search

2 December 2002

Date of mailing of the international search report

18/12/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Puonti-Kaerlas, J

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 02/00481

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 877 402 A (CARRER HELAINE ET AL) 2 March 1999 (1999-03-02) p. 26:45-28:17, example 5 ---	1-31
Y	ALLISON LORI A ET AL: "Deletion of rpoB reveals a second distinct transcription system in plastids of higher plants." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 15, no. 11, 1996, pages 2802-2809, XP001120083 ISSN: 0261-4189 cited in the application p. 2803, fig 1 ---	1-18, 24-31
Y	DE SANTIS-MACIOSSEK GIOVANNA ET AL: "Targeted disruption of the plastid RNA polymerase genes rpoA, B and C1: Molecular biology, biochemistry and ultrastructure." PLANT JOURNAL, vol. 18, no. 5, June 1999 (1999-06), pages 477-489, XP002221886 ISSN: 0960-7412 cited in the application p. 478, 482, fig 1,2 ---	1-18, 24-31
Y	MONDE RITA-ANN ET AL: "Post-transcriptional defects in tobacco chloroplast mutants lacking the cytochrome b6/f complex." PLANT JOURNAL, vol. 21, no. 1, January 2000 (2000-01), pages 61-72, XP002221887 ISSN: 0960-7412 cited in the application abstract, p. 62-63, fig 1,2 ---	1-17,19, 21-31
Y	SERINO GERMAN ET AL: "RNA polymerase subunits encoded by the plastid rpo genes are not shared with the nucleus-encoded plastid enzyme." PLANT PHYSIOLOGY (ROCKVILLE), vol. 117, no. 4, August 1998 (1998-08), pages 1165-1170, XP002221888 ISSN: 0032-0889 abstract, p. 1169-1170, fig. 1 ---	1-18, 24-31
	--- -/--	

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 02/00481

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	RUF S ET AL: "TARGETED INACTIVATION OF A TOBACCO INTRON-CONTAINING OPEN READING FRAME REVEALS A NOVEL CHLOROPLAST-ENCODED PHOTOSYSTEM I-RELATED GENE" THE JOURNAL OF CELL BIOLOGY, ROCKEFELLER UNIVERSITY PRESS, US, vol. 139, no. 1, October 1997 (1997-10), pages 95-102, XP000885779 ISSN: 0021-9525 cited in the application abstract, p. 97-98, fig. 5 ---	1-17, 19, 20, 24-31
A	SUZUKI JON Y ET AL: "Engineering of the rpl23 gene cluster to replace the plastid RNA polymerase alpha subunit with the Escherichia coli homologue." CURRENT GENETICS, vol. 38, no. 4, November 2000 (2000-11), pages 218-225, XP002221889 ISSN: 0172-8083 ---	
A	FISCHER NICOLAS ET AL: "Selectable marker recycling in the chloroplast" MOLECULAR AND GENERAL GENETICS, SPRINGER VERLAG, BERLIN, DE, vol. 251, no. 3, 1996, pages 373-380, XP002180057 ISSN: 0026-8925 cited in the application ---	
A	DANIELL H: "New tools for chloroplast genetic engineering." NATURE BIOTECHNOLOGY. UNITED STATES SEP 1999, vol. 17, no. 9, September 1999 (1999-09), pages 855-856, XP002221890 ISSN: 1087-0156 ---	
A	BOGORAD L: "Engineering chloroplasts: an alternative site for foreign genes, proteins, reactions and products" TRENDS IN BIOTECHNOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 18, no. 6, June 2000 (2000-06), pages 257-263, XP004203651 ISSN: 0167-7799 ---	
A	KOFER W ET AL: "REVIEW PEG-MEDIATED PLASTID TRANSFORMATION IN HIGHER PLANTS" IN VITRO CELLULAR & DEVELOPMENT BIOLOGY. PLANT, GAITHERSBURG, MD, US, vol. 34, no. 4, October 1998 (1998-10), pages 303-309, XP001084967 ISSN: 1054-5476 ---	
	---	
	-/--	

# INTERNATIONAL SEARCH REPORT

National Application No

PCT/EP 02/00481

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 00 32799 A (CALGENE LLC)  8 June 2000 (2000-06-08)  -----</p>	

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/EP 02/00481

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:  
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

## FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

## Continuation of Box I.2

Present claims 1-17, 19 and 22-31 relate to an extremely large number of possible compounds/products/methods. Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the compounds/products/methods claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product/compound/method by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely those parts relating to the specific examples and *rpoA*, *rpoB*, *ycf3* and *petA* genes.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/00481

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 5877402	A	02-03-1999	US 5451513 A	19-09-1995
			US 6388168 B1	14-05-2002
WO 0032799	A	08-06-2000	EP 1144665 A1	17-10-2001
			JP 2002531096 T	24-09-2002
			WO 0032799 A1	08-06-2000



**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☒ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**